

25 November 1982

Coming out of the long tunnel

British universities have weathered enforced economy better than many feared. The return to normalcy may be more testing.

In the two years since the British government told its dependent universities that they would have to share in a general reduction of public expenditure from which only the armed services and the police were excepted, many British universities have learned some unacademic tricks. Half-way through the adjustment period, due to end with 1983–84, most universities have been able to reduce their payrolls by natural wastage and because of the attractions to those older than 50 of the early retirement schemes sanctioned by the Treasury. Only when the dust has settled will it be known whether the academics who have left include an undue proportion of those who can be least spared — and whether the cost of paying for these early pensions, a charge on future budgets, will be supportable. At the same time, many universities have made deals with industry or commercial concerns that help both to maintain the scale on which research is undertaken and to shed some of the cost of the tenured payroll. Again, it is too soon to know how seriously these developments will compromise the quality of teaching — or the impartiality of the universities concerned. But especially because the research councils have also done their best to rally round, and with the prospect of extra government money for the appointment of younger people to university faculties, it is natural that academics should be looking forward to the ending of this long period of deprivation.

That temptation should be resisted. The months between now and September 1984 could yet bring trouble. The announcement last week that the University of Aston (in Birmingham) is planning to fire tenured academics is a sign that the university has exhausted the supply of academics willing to settle for early retirement. Other places could yet find themselves in the same boat. It is also likely that the universities that have been unable or unwilling to comply with the new (reduced) targets for student numbers put out in June 1981 by the University Grants Committee will find themselves in further trouble before the present adjustment period is over. The British government is unlikely to condone such cheekiness, while the grants committee will probably find itself having to punish the transgressors on the grounds that they are making an inequitable claim on national resources. Precisely how these issues are resolved will depend to some extent on the appointment of the next chairman of the grants committee. Quite apart from the administration of a shrunken budget, the successor to Dr Edward Parkes will have to shoulder two less tangible burdens — keeping up the spirits of British academics and making good the erosion of academic standards in British universities.

Structure

Grappling with these problems would be easier if the structural problems that afflict the British university system had been solved. On past form, however, that is unlikely without external intervention. William Waldegrave, the minister at the Department of Education and Science with responsibility for higher education, was right last week to remind his audience at Westfield College, London, that British universities and academics should have been worrying about the way in which the system is run at least since 1977, when Mrs Shirley Williams, then in charge of the Department of Education and Science, sympathetically spelled out her version of what was wrong. Broadly speaking, British universities ignored or denied that

warning; they have since found themselves coerced by the government instead. The intelligence that even the previous Labour government was alarmed at the rising cost of educating students from overseas evoked from British academics an opinion that to change the previous practice of equal access would be illiberal. The Labour government's imaginative offer of a system of scholarships for overseas students was not seriously discussed, with the predictable result that the Labour government's successor, the present administration, impatiently instructed British universities that fees should be paid by students from overseas. Much the same is likely to happen if British universities now continue to pretend that persisting anomalies in higher education are no concern of theirs: the government (this or the next) will intervene again.

Support

The cost of supporting students in higher education is plainly bound to be a running sore in the years ahead. Present arrangements require that local authorities should pay the tuition fees of all students accepted for places in higher education and provide them with maintenance grants as well. Local authorities recover almost all the cost of these subventions from the central government, which is why the government complains that higher education (not just universities but polytechnics and colleges of further education) has an open claim on public funds. This feature of the present arrangements for educating young people in Britain is the reason why the University Grants Committee was compelled, in June 1981, to instruct every British university that, by 1983–84, the numbers of home students should not exceed some arbitrary quota. Universities are well within their rights to argue that the consequences are often uneconomic. Universities would also be technically within their rights if they complained that the government has been slow to set up a mechanism for regulating the cost of other sectors of higher education. They will, however, be unwise if they continue to insist that their decisions on which students they are prepared to teach are financially binding on the central government; that position could easily be turned if the government decided that students would automatically be provided with maintenance grants calculated only to be enough to allow them to attend their neighbourhood university, but with a loan scheme allowing them to go further afield if they have the chance. That, or a system of competitive scholarships for potentially able students, is likely to be forced on the British universities if they find nothing better.

The structural problem occasioned by the uniformity of the British university system, and its ideological separation from the rest of British higher education, also needs attention. As is well known, British universities seek to be like Oxbridge but will occasionally settle for a different kind of self-image if it happens to be something equally distinctive, that of Imperial College, London, for example. Farcically, all British universities count themselves at least one rung higher on the ladder of intellectual opportunity than the polytechnics. That conceit, however, is mistaken. Many polytechnics provide their students with a better education (a more employable skill) than some universities. Some polytechnics are more distinguished than some universities at research. So is it wise of the universities to continue to insist that the rest of higher education is untouchable? That difficulty would



become especially hazardous for the weaker universities if, as seems probable, the British government finds a way of ridding the universities of the quota system under which they now have to labour, for then the weak among them would be known not merely in common-room gossip but from their success (or the opposite) in attracting students.

Another abiding problem of British higher education which academics stolidly refuse to contemplate is educational. British universities, insistent as they are on the equation between academic excellence and academic freedom, and used to equating freedom with the right to do what they have always done, pay very little attention to the way in which they educate their students. Prepared though they are to go to the barricades whenever the grants committee should suggest that one or other of them should abandon its degree course in agriculture or Portuguese, their willingness to delegate the selection of the students whom they will teach to a computer centre in Cheltenham is a proof that they are only half-serious. If British universities hope to survive, they must set out to choose their own students by their own devices, snapping their fingers at the cumbersome apparatus of school-leaving examinations but saying instead that they will provide a higher education for sufficiently able if ill-prepared students even if that means teaching them for four years and not just three. The government would not welcome such a development, but would not be qualified to resist.

But are British universities not autonomous institutions, whose academics collectively decide what kinds of places they should be? That is the theory, but no longer the practice. In an ideal world, or even in the United States, universities conscious that their reputations leave something to be desired would think of strengthening their claims on student and academic attention by offering good teachers attractive terms of service. In Britain, there is instead a nationally agreed scale of salaries for academics from which Oxbridge is to some extent exempt. The result is that even when some provincial university is able to create a superb department in some field, the advantage is bound to be short-lived; the people concerned will eventually be hired away to a more prestigious or congenial place. If on the other hand some university should model itself on some place that is not Oxbridge but, say, the University of Aachen or the Massachusetts Institute of Technology, it would quickly find itself regarded by its peers as a kind of jumped-up polytechnic — and would equally be prevented from keeping its academics by the uniformity of the terms of service under which it (and they) labour.

The agenda accumulating for the next chairman of the University Grants Committee is therefore formidable: how to share out a government subvention while providing those who depend on it with an incentive towards diversity, how to educate most students more broadly without sacrificing the service now provided by those universities that are already excellent, and how to make a bridge between universities and other higher education establishments without humiliating either. There follows from this a simple recipe:

- Pensioner universities should be compelled towards autonomy, told in advance what their budgets will be for as many years ahead as can be managed but then subjected to an intellectual rather than procedural review of their academic attainments.
- Universities should be reminded (some have forgotten) that the reason for their existence is the education of the young; the opportunity for research follows from that, and is not capable of being institutionalized by itself. Getting hold of suitable entrants to undergraduate courses should therefore be central to most universities' concerns (in which case the abandonment of school-leaving examinations and the disbandment of UCCA will come naturally). Teaching elementary courses (and worrying about how well they are doing) will naturally frighten British academics (in which cases they should be fired).
- Making bridges with the rest of higher education should be relatively simple: why should not the University Grants Committee provide support for the infrastructure of good research whenever it may be carried out?

Biting off others' tongues

The UK Medical Research Council seems to be irrationally sensitive about a planned clinical trial.

The blank space on page 310 is intended to be spectacular. Earlier this week, the space was occupied by a reasonable letter replying to an earlier piece of correspondence from Dr Arthur Wynn (see *Nature* 11 November, p.102) raising doubts about the British Medical Research Council's plan to carry out a controlled study of the utility of folic acid as a dietary supplement in the prevention of recurring embryonic neural tube defects, usually manifested as spina bifida in human births. After the page concerned had been readied for printing, the author of the letter asked that it should be withdrawn on the grounds that its publication would acutely embarrass the Medical Research Council, but for reasons that he did not fully understand. As it happens, the same disputed page of *Nature* had earlier on the same day been adorned with yet another item of correspondence on the same subject arguing that the disputed trials had needlessly been delayed for two years, during which time it had become apparent that the balance between the risks of folic acid supplements, and the benefits thereof, had shifted towards the latter. That letter had also been withdrawn, again at the request of the Medical Research Council.

Objectively, there should be no problem about the plan to mount a controlled study in Britain of the influence of folic acid on the incidence of neural tube defects (spina bifida) in human births. Two years ago, the UK Department of Health seems to have taken that view, calling a meeting to plan how the trial should be carried out. Delay has allowed three categories of ethical concerns to flourish — protests that if there is evidence that folic acid will prevent spina bifida, it is unfair to deny the mothers in the control group the appropriate supplement of their diet, the claim that unnaturally large amounts of folic acid in the diet may be harmful (shades of fluoride in the drinking water) and legalistic but unrealistic worries (in a country in which abortion is permissible but not entirely free) that for a mother to consent to membership of the control group denied folic acid supplement would be to infringe the rights of a child unborn or even not conceived.

None of these arguments is absolute. The case that folic acid will prevent recurrences of spina bifida is strong but not overwhelming; it would be more persuasive if there were a convincing tale to tell of how this supplement, rather than some other, were effective. The chance that folic acid might be harmful would be more persuasive if the chemical were cheaper. The challenge to the rights of the unborn child is philosophically powerful but only to the extent that putative parents sense that unborn children could be cured of putative spina bifida by a suitable dose of folic acid.

Resolving such issues patently requires nothing less than what the Department of Health had in mind two years ago — a controlled trial set up in such a way that it could be abandoned quickly. The Medical Research Council should resolve on such a course when it meets next, on 7 December. It should not be deterred by what the politicians have to say.

Meanwhile, innocent bystanders should ask themselves why *Nature* should be printing several square inches of empty space, and why the authors of the words that would ordinarily have filled them have at the last minute been frightened away from publication. In each case, the groups concerned have said that the pressure came from the Medical Research Council. By all accounts, it would be entirely unworthy to suppose that any component of that pressure is concerned with future research grants or their availability. But can it really be sensible that a research council already sufficiently entangled with muddy and muddled political issues beyond its competence should also seek (and successfully) to suppress what its own people have to say, if not openly in its defence then in the cause of helping the understanding of a tendentious issue?

Biotechnology patent challenged

Ex-colleague seeks share of the credit

Washington

Dr Robert Helling will formally press a claim of co-inventorship of the Cohen-Boyer genetic engineering patents (see *Nature* 11 November, p.95). Helling, now at the University of Michigan, worked with Dr Herbert Boyer at the University of California, San Francisco, during a sabbatical in 1972-73 and co-authored the 1973 paper in *Proceedings of the National Academy of Sciences* that forms the basis of the patents. He has consistently refused to sign a disclaimer of co-inventorship as requested by Stanford University and the University of California, but decided only last week to press his claim actively.

The decision seems likely to delay further the issuing of the second Cohen-Boyer patent, at present in limbo after the patent office tentatively rejected it, in part because of the unresolved role of Helling. It may also increase pressure for a re-examination of the first patent, issued in December 1980.

The decision last week came after the University of Michigan agreed to take up the case on Helling's behalf. James Dautremont, the university's intellectual property counsel, said the matter would be discussed with both the patent office and the two universities involved "in a completely cooperative effort to determine where the legally proper inventorship lies".

It is not clear whether Helling or the University of Michigan has a financial

stake in the outcome. Helling did sign a patent agreement with the University of California covering his term there that appears to assign any patents to that university; if that is so, all he could gain would be the recognition of having his name added to the landmark patents. On the other hand, if it turns out that he was covered by the University of Michigan's patent regulations at the time, he would divide his share of the royalties equally with the University of Michigan.

In the response of 1 November to the patent office's proposed rejection of the second patent, the attorney for Stanford and the University of California argued that Helling's failure either to claim or to disclaim co-inventorship in the eight years since the patents were filed should not

be permitted to hold up the patent. But Dautremont counters that "inventorship can be challenged or modified as appropriate according to the facts at any time". The question, he says, is whether "Professor Helling in fact made a significant contribution to the invention as recited in the claims of the patent. I'm sure that will become clear from written records, lab notebooks, and so on".

Dautremont emphasized that "we're not in a fight at all" and said he expected the matter to be resolved "amicably". And he suggested that it would be in the best interests of Stanford University and the University of California to add Helling's name to the patent if the facts warrant it in order to remove a "cloud over this issue".

Stephen Budiansky

Strike response to French reform

The plans of M. Alain Savary, French minister of national education, to set in motion what he described in early October as a "global reform" of the universities and their prestigious sisters, the *grandes écoles*, seem to be meeting with an equally global resistance.

The university trade union SNE-Sup (Syndicat National de l'Education Supérieure) has this week, therefore, called a two-day national strike, demanding that the reform be given "new life"; while the *grandes écoles*, engineering schools well represented in the government administration and traditionally regarded as providing the best education in France, have been applying their own behind-the-scenes pressure to ensure that real life does not affect them. Given M. Savary's gentlemanly approach to politics, the result

may be something closely approaching the *status quo*.

Meanwhile, the water is being muddied by the ministry, which appears reluctant to reveal exactly what the minister has in mind. First, there is the unpublished — but circulating — "Jeantet report", described by conservatives as the work of a "radical dreamer". It foresees the absorption of some *grandes écoles* by the universities, and attacks elitism. It is the result of M. Claude Jeantet's questionnaire to the universities, *grandes écoles* and other institutions and was intended to form the basis of the reform, but it now it seems the minister is distancing himself from it.

Then there are three other texts, all published within two weeks in October, which purport to outline the minister's views. Two of these are speeches by Savary himself, and one — the last — is an "information note" from the ministry. All are partly contradictory. They appear to indicate an evolution towards conservatism; but the information note fails to touch on many key issues — such as research, or reform of higher degrees — and leaves room for the reform to be radical in some respects. (For example, universities are defined as including "schools" — by implication the *grandes écoles* — but *grandes écoles* are also defined outside the universities; thus *grandes écoles* directors are asking themselves which kind of school their own will be.)

As if further to confuse the issue, the director-general for higher education and research at Savary's ministry, the newly-appointed and activist M. Jean-Jacques Payan, said last week that he had been taken fully into Savary's confidence over the final shape of the reform and that it will reveal "many new things" when it is published, he predicts, on 10 December. For example, he says, the reform will allow

IIASA struggles on in hope

The International Institute of Applied Systems Analysis (IIASA), the East-West systems analysis centre in Vienna, has had a reprieve from the United States, and a partial reprieve from Britain.

In both countries, the member institutes of IIASA — the National Academy of Sciences in the United States and the Royal Society in the United Kingdom — had withdrawn as of 1 January 1983, and the problem was to find new members that could pay the subscriptions for IIASA's total budget of around £5 million (in 1981). Now the American Academy of Arts and Sciences has agreed to take over the US membership, raising the money needed from private foundations and industry.

In Britain, the situation is less clear. The Fellowship of Engineering, a body with few funds of its own, is debating whether to join IIASA, but it will have to

find the whole subscription outside — either in industry or in government departments other than the Department of Environment, which originally paid the bill and whose lack of interest in IIASA led to the Royal Society's withdrawal.

Meanwhile, IIASA — crossing its corporate fingers — has drawn up its research plans for 1983. Project titles include: patterns of economic structural change and industrial adjustment; national agricultural policies; energy development, economy, and investments; structural change in the forest sector; institutions and environmental policies; population — ageing and changing lifestyles; integrated regional and urban development; adaptation and optimization; and negotiations and interactive decision making.

Robert Walgate

all universities to teach engineering, and grant *Diplômes d'ingénieur*, previously the jealously guarded preserve of the *grandes écoles*, and not previewed in any ministry text so far.

The next step, however, is the presentation of a near-final text to the advisory Conseil National de l'Éducation Supérieure et de la Recherche (CNESER, the national council for higher education and research) which was scheduled for Thursday 25 November — the first day of the planned SNE-Sup strike. However, the unions have requested that the CNESER meeting be postponed, and on Monday a new date had not yet been arranged. CNESER is reported to be very closely

divided between radicals and conservatives, and may vote for a text close to Jeantet — which would set the cat among the *grandes écoles* pigeons, unless Savary were entirely to ignore CNESER's advice — something that would be politically inadvisable. CNESER, in fact, along with M. Payan himself — who is *ex officio* president of CNESER — may be the main element of volatility in a situation long since frozen by the traditional conflicts between the *grandes écoles* (broadly on the right) and the universities and unions (broadly on the left). Whether the strike will influence either CNESER or Payan is still to be seen.

Robert Walgate

West Bank universities

Request for pledge withdrawn

The Israeli government has abandoned an earlier regulation demanding that foreign lecturers working in universities and colleges on the West Bank should sign a pledge not to assist the Palestine Liberation Organization (PLO). The announcement that the regulation would be suspended (and unofficial hints that it could be formally rescinded in the near future) came a few days after a swingeing attack by US Secretary of State George Shultz, who last Thursday called the regulation a threat to academic freedom reminiscent of the McCarthy era in the United States.

Israeli officials nevertheless deny that the events are related. Instead, they suggest the relaxation was made possible by quiet diplomacy between the civil administration of the West Bank and the university authorities. The only purpose of the regulation, they stress, had been to preserve the function of the universities as centres of higher learning and to avoid their politicization.

It is clear, however, that the Israeli government was somewhat taken aback by the strength of the international reaction to its move. One senior official reportedly expressed "surprise" at Mr Shultz's appeals to Israeli academics to join in the protests. In fact, the Israeli academic community has on several occasions come out in support of the West Bank universities — in November 1981, when the Bir-Zeit University in Ramallah was temporarily closed, a group of lecturers and research students at the Hebrew University of Jerusalem attempted, perhaps cosmetically, to organize fill-in courses for students who otherwise would have had to interrupt their studies.

The constitution of the West Bank universities and colleges under Israeli administration is complex. At the time of the Israeli take-over, there were three such establishments, Bir-Zeit (near Ramallah), El-Najah (near Nablus) and the Kadouri Agricultural College (at Jenin). These were accorded full academic status by the

authorities (Bir-Zeit College became a university in 1975) and two further colleges were founded, the Islamic College in Hebron and the (Catholic) College-Frères (now the University of Bethlehem). All these are financed, indirectly, by the Israeli government, through its budget for the occupied territories, which is distributed by the civil administration of the West Bank.

Just how many lecturers refused to sign the pledge is not clear — Mr Shultz put the figure at around 120, of whom, he said 22 have already been expelled from Israel and 30 others suspended from teaching, while others were told that they would be required to leave the country when their three-month tourist visas expired. (This latter category presumably refers to new arrivals who took up their teaching posts in October this year). The Israelis, on the other hand, claim that only three lecturers were actually expelled, and that the rest were merely told that their residence permits were being withdrawn. They admit, however, that even to the newest arrivals the pledge was something that they had not foreseen when they accepted the post, and that the lecturers' refusal to sign the pledge was inspired not by a desire to assist the PLO but as a matter of principle.

"Freezing" the pledge, in the case of lecturers, however, could make difficulties with other foreign workers on the West Bank, all of whom, a government spokesman announced last week, were required to sign the pledge and had already done so in "hundreds if not thousands".

Vera Rich

International conferences

Anti-Israel moves

Rehovoot

Recent events in Lebanon threaten seriously to undermine relations between Israeli scientists and their colleagues overseas. This is one reason why Israeli scientists were relieved to hear from Weizmann Institute physicist Shalheveth

Freier that despite a prolonged and often acrimonious debate on events in Lebanon at the recent Pugwash meeting in Warsaw, the organization did not adopt an anti-Israel resolution.

Instead, Pugwash adopted, after a full 20 hours of discussion in working group and plenary sessions, a far more balanced resolution than seemed possible at the outset. The resolution called for "the withdrawal of all non-Lebanese forces and the creation of a strong Lebanese government acceptable to all factions".

Freier's reception in Warsaw was thus less hostile than that of many other Israeli scientists attending international meetings in recent months. Thus one young Israeli neurobiologist arrived at a scientific conference at a French university to find a sign posted outside the cafeteria which read: "The Israelis are assassins". Many delegates echoed this view and flatly declared that they would never visit Israel again so long as the country remained "aggressive".

Strangely enough, the neurobiologist (from Tel Aviv) says he found greatest understanding from a Syrian scientist who had once lived in the city of Homs, and who was all for "smashing the PLO and the Syrian Army", presumably because several members of his family had been among the 10,000 people killed when the Assad government put down an uprising of devout Moslems in that city last year.

Some Israeli scientists have even found themselves "disinvited" from meetings during the summer and autumn. One chemist, for example, has received the following communication from a colleague in Europe: "The current situation in Lebanon is causing deep concern here. In particular, the recent actions waged by Israeli forces against the city of Beirut appear cruel and unnecessary. For that reason my group is unanimous in feeling that we cannot maintain your visit here as planned. Please understand that this is not directed against you personally."

In his reply, the Israeli chemist said that his erstwhile hosts had "the unquestionable right" to cancel the invitation, but he wondered whether they had also boycotted Iraqi scientists in the wake of their government's brutal attacks on the Kurds, Palestinian Arab scientists after the massacre of Israeli bus passengers near Tel Aviv and Israeli schoolchildren in the Galilee, or Soviet scientists because of events in Afghanistan and Poland.

Many overseas researchers seem also to have boycotted conferences held here in recent months. Thus there were only 150 participants (instead of the expected 250) at the International Symposium on Recent Developments in Perinatal and Childhood Infections and 800 participants (against 1,000 expected) at the Sixth International Congress on Hormonal Steroids. Many bodies that were thinking of Israel as a conference site for 1986-88 have also, after the furore over Lebanon, altered their plans.

In this situation, Israeli scientists find themselves fighting two battles at the same time. On the one hand, they are trying to convince their overseas colleagues that the common judgement of Israeli policies is too harsh. On the other hand, most of them are trying to convince their fellow citizens of the need to change many of these policies.

Prominent scientists — including Professor Ephraim Urbach, president of the Israel Academy of Sciences and Humanities, and Professor Ephraim Katzir, former President of the State of Israel — played a prominent part in the successful attempt to force the appointment of a commission of judicial inquiry to probe the massacre of

Palestinian refugees in Beirut. Many of these same scientists actively oppose the don't-give-back-an-inch views of the Begin government.

Even they however, are split over the question of a separate Palestinian state. Some think the emergence of such a state inevitable (or even desirable), while others would agree with what Shalheveth Freier said at the Pugwash meeting in Warsaw, where he declared: "If the general atmosphere in the Middle East continues to be uncompromisingly hostile to Israel, I suppose I should feel compelled to resist the creation of another hostile state. If, however, peace were to descend on this area, I believe that all honourable options would be open." **Nechemia Meyers**

Academic consultancy

Mass. General placates Hoechst

Washington

Massachusetts General Hospital (MGH) is going to unusual lengths to ensure that faculty consulting with outside firms does not conflict with the terms of its 10-year \$50-million agreement with Hoechst AG, the German chemical company.

In the case of one faculty member just appointed to the department funded by Hoechst, MGH is demanding that his consulting with other firms be on a non-confidential basis, a condition apparently not required of other MGH faculty.

The 1981 agreement between Hoechst and MGH represents the largest joint venture so far between industry and an academic institution. Hoechst agreed to provide the money for a new department of molecular biology in exchange for an exclusive licence to any patents that result from the department's research. The agreement also requires any faculty collaboration or consultation with for-profit firms to be cleared with Hoechst.

MGH has now apparently taken it upon itself to negotiate the particulars of consulting contracts that molecular biology faculty members have with outside firms to ensure that they are acceptable to Hoechst. In the case of Dr Brian Seed, the newly-appointed faculty member, MGH has engaged the services of a prominent Boston law firm, Ropes and Gray, to negotiate changes in his consulting arrangement with Genetics Institute. Genetics Institute, the spin-off from Harvard University's short-lived plan to form its own profit-making genetics engineering company, is negotiating through its own prominent Boston law firm, Hill and Dorr.

The MGH lawyers are demanding that since Seed is free to pass on information from his Hoechst-supported work at MGH to Genetics Institute, he should similarly be free to pass on to Hoechst any information gleaned through his consultations with Genetics Institute. Seed, who says he has heard nothing about the current status of the negotiations, says the basis of MGH's

position is Hoechst's concern over its ability freely to exercise its patent rights under the agreement: "Hoechst is worried that as a result of my consulting for another company I might take up in my lab some work based on proprietary information". Thus Hoechst might end up supporting some research that it could not patent.

MGH officials deny that their actions reflect any special policy towards the department of molecular biology or Hoechst. "We want to make sure that their consulting activity is consistent with the hospital's policy", says Dr Ronald Lamont-Havers, deputy general director for research. "Any consultative agreement needs to be cleared with the hospital to ensure that there is no conflict of interest."

But Lamont-Havers did acknowledge that while the hospital has advised other faculty members that their consulting contracts need to be changed, it has only actively negotiated those changes in the case of department of molecular biology faculty. And he appeared surprised to learn of the non-confidentiality requirement that MGH's own lawyers are demanding in Seed's case. "Usually, in our consulting agreements, we expect that they would receive proprietary information and would not be free to discuss proprietary information", he said.

Dr Howard Goodman, director of the department of molecular biology, denies that anyone in his department has been required to give up any consulting or that appointments have been made contingent upon modifications in an appointee's consulting contracts. And Seed seems confident that an accommodation will be reached in his case. "I doubt that my relationship with Genetics Institute will be severed", he says. But the lingering question is whether the conditions being demanded by MGH — and which Genetics Institute reportedly considers unacceptable — will make it impossible for at least some faculty consulting arrangements to continue. **Stephen Budiansky**

US defence research

Computer plan

Washington

The US Department of Defense (DoD) is adding to its existing investment in computer research several new programmes designed to counter possible Japanese domination of the field. The budget for fiscal year 1984, which is now under negotiation behind closed doors, is likely to include hundreds of millions of additional dollars for special programmes in super-computer research and software. DoD is already supporting research in Very Large Integrated Circuits, the technology of gallium arsenide as a replacement for silicon, and other computer-related programmes.

In a speech to a professional meeting in Orlando, Florida, Robert S. Cooper, director of the Defense Advanced Projects Research Agency (DARPA) announced that he would counter the Japanese computer effort with a research programme aimed at achieving the extraordinary speed of 10,000 million floating point operations per second by 1990. Present "supercom-



puters" such as the Cray III and Control Data's Cyber 205 attain speeds of 100 megaflops, while the Japanese programmes aims at achieving 1,000 megaflops. DARPA's programme is rumoured to have initial costs of \$150 million.

DARPA supports basic research and exploratory development for the directorate of Defense Research and Engineering and would not, therefore, be involved in applications. Possible defence applications include warhead delivery systems capable of "deciding" for themselves which targets to attack, and thus not dependent on vulnerable telemetry systems.

There is also to be a substantial new programme in the development of software to be managed by a new office to be established within the Defense Research and Engineering directorate. According to a report from the office of Dr Edith Martin, the deputy under-secretary at the Pentagon with responsibility for research and engineering, it is planned to spend an extra \$30 million on software development in the financial year beginning on 1 October 1983, to provide further funds in succeeding years and to establish a military software institute. It is estimated that between \$5,000 million and \$6,000 million worth of software is already "embedded" in defence systems, and that the amount will rise to \$32,000 million by 1990.

Increased funds are also likely to be

made available for the Very High Speed Integrated Circuits programme as well as for the research and development programme based on gallium arsenide, a material that offers faster semiconductor devices and may be more resistant to disturbances such as electromagnetic pulses.

The Pentagon's efforts to match the Japanese effort in advanced computers is mirrored by two initiatives by the computer industry. However, the plan of 15 computer companies under the leadership of the Control Data Corporation to invest between \$50 and \$100 million a year in software and hardware development has not yet become a reality even though the founders are confident that the US Department of Justice will decide that the proposed consortium, the Microelectronics and Computer Technology Corporation, does not violate anti-trust laws.

The non-profit Semiconductor Research Corporation, set up by 13 computer companies earlier this year, has however begun making grants to academic institutions considered to be centres of excellence in computer development. Last week, the corporation announced grants for developments in large-scale integrated circuit technology to Cornell University, the University of California at Berkeley and the Carnegie-Mellon University.

Deborah Shapley

French win contract

The British Central Electricity Generating Board (CEGB) has commissioned the design and safety study of the pressure vessel for the planned Sizewell B pressurized water reactor (PWR), Britain's first, from Framatome, the main constructor of the 40 or so French PWRs.

Thus Dr Walter Marshall, now director-general of CEGB, has proved true to his word. In March, when he was chairman of the United Kingdom Atomic Energy Authority, and head of the PWR task force that tidied up the Sizewell safety design, he was questioned about the problems that Framatome had had with cracks under the pressure vessel cladding. This would be just the time to buy from the French, said Marshall — because "they have been caught with their pants down". The problem was one of heat treatment, and has now been solved, Marshall believed, probably leaving Framatome with a better understanding of such vessels than other manufacturers.

From his position as head of CEGB, Marshall has now approved the offer of the design contract, worth £213,000, to Framatome. This will not be complete until May 1983, after the January 1983 Sizewell inquiry, but — according to Framatome sources — a report amounting to "the bulk" of the study will be ready in time for the inquiry.

Robert Walgate

MRC annual report

Universities stand to gain

Like publishers, heads of research councils are usually a gloomy bunch when it comes to public statements on the financial position of their organizations. But at last week's launch of the annual report of the UK Medical Research Council (MRC), the council's secretary, Sir James Gowans, was remarkably cheerful about MRC finances and even offered a helping hand to the universities.

The hand-out, which all told could amount to 1 per cent of the annual budget of MRC, is offered to university research teams, in particular those already within the system of dual support by MRC and university, whose cutting edge has been dulled by diminished support from the university. To restore their competitiveness until such time as university finances are again healthy, MRC is offering to convert such teams into MRC Groups, providing them with up to £50,000 per annum.

The scheme, advertised earlier this year, has attracted some twenty applicants, almost all of them already substantially financed by the MRC. To ensure that the money is awarded to cases of genuine hardship, the vice-chancellor of the university has to make the application and to promise that he is not trying to free resources which will be redeployed into unrelated activities. Although no decisions have yet been made it is likely that the support awarded to MRC groups will cover such items as radiochemicals, an electron microscopist, an animal-house technician or even a departmental secretary.

MRC seems willing to spend up to £1 million a year in this way. Where will the money come from? Nowhere in particular, according to Sir James Gowans. Nevertheless, two main sources seem likely.

The first would be the £1 million that would be saved were MRC to withdraw its support of the European Molecular Biology Laboratory (EMBL) in Heidelberg. The suspicion that it might do so arises from an instruction from the Advisory Board for the Research Councils telling MRC to review its participation in EMBL. Asked why he thought the review was called for, Sir James Gowans would say only that the question should be addressed to the Advisory Board — of which he is a member.

Alternatively, it might be surmised, MRC could support its proposed groups by spending less on project and programme grants to university teams. According to the annual report, "the growing volume of applications for support . . . suggests . . . that some good work will continue to go unfunded" but in fact no really good project ever seems to fail to be supported.

Looking ahead, Sir James Gowans drew attention to the review next year of the five-year agreement between MRC and the biotechnology company Celltech. The agree-

ment runs until 1985 and Sir James thinks "it is going all right". He also mentioned the National Institute of Medical Research (NIMR) which this year will consume about £9 million of the £106 million MRC budget. Sir James expects Dr D.A. Rees, formerly principal scientist at Unilever's Colworth laboratory but director of NIMR since 1 October, to increase the links between NIMR and industry and to open facilities at NIMR to university researchers. In addition, the structure of NIMR will be reorganized during 1983.

Peter Newmark

Rostow a "dove"

Washington

Part of the reason for the apparent stalemate of the Geneva arms control negotiations seems to be a dispute between a group of conservative senators and Mr Eugene V. Rostow, director of the Arms Control and Disarmament Agency (ACDA). The dispute may come to a head as soon as 29 November when a group in the Senate led by Senator Jesse Helms (Republican, North Carolina) will try to send Rostow a warning that he is too dovish towards the Soviet Union — and put the President on notice as well.

The Helms group first signalled its discontent in March, when the Senate Foreign Relations Committee considered the nomination of two long-term ACDA officials kept on by the Administration at Rostow's urging. Helms complained that Robert Grey, nominated as deputy director, and Norman Terrill, nominated as assistant director for nuclear weapons policy, were hold-overs from the Carter Administration and too dovish.

Since then, confirmation of the two nominees has been held up in the Senate because of Helms's objections. When Congress reconvenes on 29 November for about a month, there will be a move to bring the nominations to the floor of the Senate, which is possible only if Helms withdraws his objection. There is a rumour that only the Grey nomination will be called up. The Helms faction, which would prefer General Edward Rowney to have Rostow's job, seems to have calculated that postponing Terrill's confirmation as acting assistant director will continue to embarrass Rostow and, in particular, prevent him from taking a decisive position on arms control.

If the two nominations are not confirmed by the time Congress finally disbands, the whole nomination process will have to begin all over again in the new Congress next February.

Deborah Shapley

Soviet psychiatric abuse

Allegations met by red tape

The Soviet All-Union Society of Psychiatrists (AUSP) has adopted a new approach to accusations by human rights groups about the confinement and "treatment" of dissidents in Soviet mental hospitals. Instead of rejecting all such allegations out of hand, it has supplied the World Psychiatric Association (WPA) with details of two alleged cases and has promised information on four more.

The fact that the data appear incomplete, and the extreme slowness with which they have been made available, have evoked considerable suspicion among some members of WPA. Is this a genuine change of heart or simply a delaying tactic on the part of the Soviet Union to forestall moves by several member bodies — including the Royal College of Psychiatrists and the American Psychiatric Association — to have AUSP expelled or suspended from WPA?

This is not the first time that AUSP has professed itself willing to cooperate with WPA over allegations of psychiatric abuse. During the last world congress, in Honolulu in 1977, the Soviet delegate to the general assembly handed round a substantial document containing "confidential" clinical data on twelve dissidents whose cases had frequently been cited as "alleged victims of psychiatric abuse", in the hope of convincing the delegates that the dissidents were in fact suffering from severe psychoses. The Honolulu congress narrowly passed a motion of censure against the Soviet Union, and set up a special review committee to monitor allegations of political use of psychiatry.

AUSP, however, has never acknowledged the competence of this review committee, and the executive committee has had to assume much of the responsibility for investigating allegations involving the Soviet Union. The work of the review committee so far was presented at an executive council meeting in Marrakesh earlier this month in a special report by the secretary general, Professor Peter Exner, which was reportedly condemned by the Soviet observer Professor Marat Vartanyan as being "too negative". For those member societies that have submitted allegations of psychiatric abuse to WPA, follow-up of the original complaint is hindered by what the complainants see as an unreasonable insistence on professional confidentiality.

Whether this strict insistence on professional ethics is a result of Soviet pressure is not clear. Ironically, in many cases, the complaints have been originated by the victims themselves asking to have the diagnoses made public, in order to reveal their dubious medical basis. There are growing fears among member societies of WPA that the executive council's over-

scrupulous stance on confidentiality may unwittingly be going against the interests of other individuals whose cases are under review.

Vera Rich

Romanian emigrants

Cash demands

A decree promulgated earlier this month by the Romanian Council of Ministers seems certain to hamper the movement abroad of Romanian scientists. The decree requires that Romanian emigrants should repay (in hard currency) the costs of their secondary and higher education.

The decree is reminiscent of that in the Soviet Union some ten years ago designed to recover higher education fees from Jews wishing to emigrate to Israel. The Soviet ruling evoked widespread protests abroad, including the imposition of credit restrictions by the United States, and was allowed to lapse less than a year later.

The Romanian decree is likely to attract even more vehement criticism. Already the US State Department has called it a "draconian measure", contrary to the letter and spirit of the Helsinki accords, and has hinted that it could hamper the renewal, next year, of Romania's recently acquired "most favoured nation" status.

The condition that higher education costs, however calculated, should be repaid in hard currency, will be virtually impossible to satisfy. (It is illegal for Romanian citizens to have hard currency in their possession.) The most probable objective of the decree is to clamp down on emigration, a conclusion borne out by the reports from Bonn that the Romanian government has given notice to end the agreement under which up to 10,000 Romanians of German origin are allowed to emigrate each year.

The effects of the new decree, moreover,

EEC research council

Cash injections planned

Brussels

Despite the row over the budget for Super-Sara, the project for investigating what happens when light-water reactors lose coolant (see *Nature* 11 November, p.99), the November meeting of the EEC science ministers approved a near-record number of research programmes. With one or two minor reservations the pilot stage of the Esprit programme on long-range research on information technologies (see *Nature* 3 June, p.352), the 4-year indirect action programme in favour of developing countries, a programme for the development of a translation machine and finally the experimental phase of the project to stimulate the European

are liable to be felt in all countries that have scientific exchange programmes with Romania. The decree, for example, lays down that a scholar allowed to travel to an international conference who fails to return on schedule will be treated as an illegal emigrant, and will be sued for the cost of his or her education both at home (presumably with the confiscation of assets) and in the new country of residence. The relevant clause of the decree is phrased ambiguously, and could be construed to apply retroactively to those who have left the country in recent years, especially since the mid-1970s.

Vera Rich

British Astronomer Royal



Professor F. Graham Smith, now the director of the Nuffield radio-astronomy observatory of the University of Manchester at Jodrell Bank and until earlier this year director of the Royal Greenwich Observatory, was this week appointed Astronomer Royal of England. He succeeds Sir Martin Ryle, who resigned the post as from October. One of the ironies of the new appointment is that Sir Martin's appointment as Astronomer Royal was intended to mark a separation of that post from the directorship of the Greenwich Observatory.

elaborating formal programmes. The experimental phase will test out this concept in seven areas which are considered important and in need of "stimulation" — pharmacobiology, solid state physics, optics, combustion, photometry, photoacoustics, interface phenomena and climatology.

Under the new system, a consultative committee (CODEST) made up of outstanding authorities in science and technology will advise the Commission on how to spend the money. Applications for research grants, subsidies for laboratory twinning, development contracts, subsidies for research teams, seminars and workshops will all be considered.

If the experimental phase is successful, the scheme will be extended for the years 1984–87 and a figure — perhaps as much as £12 million, or 5 per cent of the Community's joint annual research expenditure, will be set aside each year.

The scheme was first agreed in principle at the last Council on 30 June, as was the Esprit programme, for which a pilot stage was agreed by the November Council, with 11.5 million ECU (£6 million) available from the EEC budget. Universities and companies will be expected to contribute at least 50 per cent of the cost if they seek support in carrying out research. Money has been set aside for pilot projects in advanced electronics (two projects), software technology (three), advanced information processing (three), office automation (four), computer integrated manufacturing (three), and information exchange systems. Each contract must contain at least one industrial participant.

A 5-year programme budgeted at 16 million ECU (£8.5 million) will now also start, aimed at building an advanced translation machine to "extend the frontiers of computational linguistics". The advantages of such a machine are evident when one considers that about a third of the 16,000 EEC civil servants are currently involved in translating or interpreting the seven Community languages.

And for the first time, the EEC will have a joint research programme in aid of developing countries (see *Nature* 15 July, p.218); 40 million ECU (£21 million) will be spent on two sub-programmes between 1982 and 1986: tropical agriculture and medicine, health and nutrition in the tropics. The programme has raised doubts in the minds of some delegations and at the European Parliament, since despite its title the programme will mostly benefit laboratories in the EEC rather than directly helping developing countries. However, the agreement to have the programme at all is an important step towards realizing the ambition of Viscount Etienne Davignon, European Commissioner with responsibility for research and development, to make work dedicated to assisting the developing world one of the seven pillars of the EEC's research and development strategy for the 1980s. **Jasper Becker**

French research and development

Can the good times last?

France's foreign debt is now estimated to be FF 300,000 million (£25,000 million), double the figure of a year ago, and the balance of trade deficit was running at FF 12,000 million (£1,000 million) a month in September. But still France continues to promise to spend, spend, spend on research and development. Earlier this month the National Assembly voted the government civil research budget for 1983: it will be FF 52,200 million (£4,300 million), 28 per cent up in money terms over 1982, and 17.8 per cent up in real terms. This is not a jot different from that previewed in the July "law for research" but the economic problems are raising fears that the figures will prove, in the end, a mere façade.

For instance, this year the finance minister ordered a freeze on government spending which — so far as science was concerned — held back a quarter of the planned budget for new research equipment. Caught unawares, laboratories which had already made orders found themselves falling rapidly into the red — until the minister of research and industry was able to claw back the 25 per cent just a

few weeks ago. Other ministries, such as the ministry of national education, were not so successful. There is talk next year of a freeze of 30 per cent. Will the research minister claw that back too? Some doubt it, despite the fact that in France, research and technology appear to have become the new faith.

Nevertheless the budget figures are worth analysing as indicators of intention. Jean-Pierre Chevènement, the research and industry minister, plans to cut his cake of FF 52,200 million (£4,300 million) for 1983 into FF 32.5 million for civil research and development, and FF 19.7 million in support of his industrial and energy policy.

The research budget proper — money for the research councils — will be up 17 per cent to FF 23,500 million. The figure includes many salaries. There will also be fiscal support to companies undertaking more research (in particular a rather back-handed relief from a new "tax on fortunes"). This and other measures are intended to help industry boost its own research budget by 8 per cent next year, as envisaged in Chevènement's grand plan.

Basic science is not the principal beneficiary of the increases, although it is not neglected. The French government's fundamental objective is the renewal of industry through long-sighted, government-managed investment. So the greatest increase — 62 per cent — within the FF 32.5 million goes to Chevènement's seven "*programmes mobilisateurs*", which cover new energies, electronics, biotechnology, research and innovation for the Third World, research on the conditions of labour, the promotion of the French language in science and technology, and general support for industrial innovation. These are the workhorses of Chevènement's policy, although nobody seems sure exactly how the cash will be harnessed to research. **Robert Walgate**

French civil research and development budget (thousand million FF)

	Budget		Increase (per cent)
	1982	1983	
" <i>Programmes mobilisateurs</i> "	5.3	8.6	62
Applied research	3.7	4.5	22
Technology programmes (nuclear, aerospace and oceans)	6.6	8.1	23
Other	5.1	5.5	8
Total government civil research and development	25.4	32.5	28

FF 11.75 = £1. Amounts are in current francs; inflation cuts increases by about 10 per cent.

Direct support for French scientists and the research organizations (thousand million FF)

	Budget		Increase (per cent)
	1982	1983	
Direct ministerial support	1.16	1.49	28
Centre National de la Recherche Scientifique	5.95	6.94	17
Commissariat à l'Energie Atomique	5.26	5.85	11
CNES (space)	2.15	2.74	27
INRA (agriculture)	1.51	1.74	15
INSERM (medical)	1.04	1.25	21
ANVAR (transfer of research to industry)	0.83	0.99	19
ORSTOM + GERDAT (Third World)	0.76	0.89	17
ADI + INRIA (informatics)	0.44	0.48	9
CNEXO (oceans)	0.40	0.49	22
AFME (new energies)	0.30	0.39	30
Institut Pasteur (Paris)	0.14	0.17	21
Other	0.11	0.12	10
Total	20.05	23.54	17

In vitro fertilization

MRC tests the climate

A set of guidelines that gives qualified approval for researchers to perform experiments on human embryos obtained through *in vitro* fertilization was issued last week in a statement by the Medical Research Council (MRC). The guidelines are likely to please scientists working in the area, but the British Medical Association (BMA) has expressed concern that they may be too liberal for its members.

The MRC statement, published in the *British Medical Journal*, states that clearly defined research on embryos which are not intended for return to the uterus is ethically acceptable if it is "directly relevant" to clinical problems. It also allows the deliberate fertilizing of human ova *in vitro* for specified and approved research projects, as well as the use of fertilized ova left over from therapeutic programmes, provided that informed consent is obtained from both parents.

The guidelines were drawn up by an MRC advisory group under Professor Geoffrey Dawes. They are intended to apply only to MRC-supported scientists, who must in any case obtain specific approval for any proposed experiment from local scientific and ethical committees. Nevertheless, MRC's ideas are likely to be influential as a basis for discussion within the numerous other committees set up to consider the implications of recent developments in the field of human fertilization, such as the British government's Warnock committee (see *Nature* 29 July, p.408).

The MRC proposals allow *in vitro* culture of human embryos only up to the implantation stage, a clearly defined phase of development corresponding roughly to the time at which embryonic tissues become distinct from those of the placenta and organs start to form. This stage is reached at 13–14 days after fertilization, considerably older than the 9-day-old embryos that were at the centre of a recent storm after Dr Robert Edwards announced that he had kept and observed "spare" embryos initially produced for reimplantation. The new guidelines also permit experiments on interspecies formation — valuable for studying defective sperm — but in this case it is recommended that embryos should not be allowed to develop beyond the early cleavage stage. Experiments on techniques for freezing embryos for storage will be allowed provided that embryos are not stored for future unspecified research.

Dr Anne McLaren, director of MRC's Mammalian Development Unit at University College London, and a member of the advisory group that drew up the guidelines, has pointed out that research

aimed at increasing the safety and the success rate of "test-tube baby" programmes is concerned with the embryo before implantation; workers in this area should not therefore find the guidelines overly restrictive.

The BMA, while accepting the MRC guidelines as "a valid contribution to the debate", appears to have reservations about how acceptable they will be to its members: a spokeswoman expressed the view that the MRC guidelines "will do nothing to allay public fears".

The BMA has its own committee under the chairmanship of Professor Peter

Quilliam, seeking to establish guidelines for the medical profession, and is unwilling to make specific comments until the committee has made its report; however, it seems possible that the adoption of the implantation stage as what is in effect a criterion for the start of a human life could be a point of disagreement. In the meantime, BMA members are not banned from involvement in MRC projects. There is clearly a lot of arguing still to be done: in the foreseeable future there are going to be strong reasons for wanting to experiment on embryos considerably beyond the implantation stage.

Tim Beardsley

Research article triggers dispute on zeolite

Washington

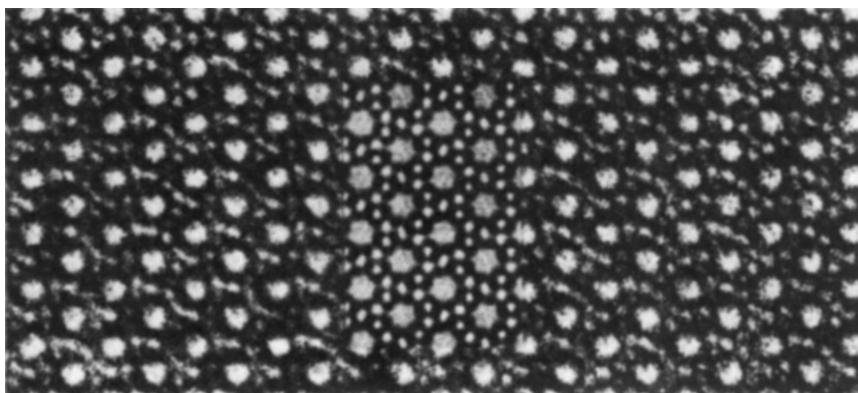
An experimental result published in the 8 April 1982 issue of *Nature* has set off a legal battle between Union Carbide and Mobil Oil that may involve hundreds of millions of dollars in patent royalties.

The dispute is over synthetic minerals known as zeolites, which have become increasingly valuable as catalysts in the production of chemicals and liquid fuel from coal. The zeolites, which are crystalline aluminosilicates, have large regular pore structures making them highly selective in binding molecules of a desired size.

In 1977, Union Carbide obtained a patent for what it claimed was a novel form of zeolite called "silicalite". But according to the analysis reported in the

to provide a copy of its response and counterclaim, which are part of the public record. But an industry expert on zeolites gave *Nature* the following explanation of the facts surrounding the case. Mobil's ZSM-5 patent covers zeolites with silicon-to-aluminium ratios from 10 to 4,000. Union Carbide claims that silicalite is 100 per cent silica within the zeolite framework; any aluminium that happens to be present in silicalite, Union Carbide says, is outside the framework, and thus does not play a part in silicalite's essential properties.

What Fyfe *et al.* showed, however, is that aluminium is present in silicalite in at least two distinct tetrahedral environments — which means that aluminium is



Mobil's ZSM-5 zeolite. The diameter of the largest pores, lined with active sites, is 5.5 Å. A computer-simulated image is inset. (Photo: G.R. Millward/J.M. Thomas, University of Cambridge.)

Nature article (C.A. Fyfe, J.M. Thomas *et al.*, *Nature* 296, 530), silicalite is in fact "identical" to a family of zeolites patented by Mobil in 1972. Mobil's zeolites, known as ZSM-5, are vital to Mobil's methanol-to-gasoline process.

Mobil then informed Union Carbide that it considered silicalite an infringement on its patent; Union Carbide responded on 21 September by suing Mobil in the Federal District Court in New York City, asking for a declaratory judgement that silicalite does not infringe on Mobil's patent. In a counterclaim filed 2 weeks ago, Mobil officially entered its charges of infringement.

Neither side is willing to discuss the case in any detail; Mobil has refused even

in the framework. Aluminium outside the framework is octahedral. "The silicalite structure is shown to be essentially indistinguishable from that of the siliceous zeolite catalyst ZSM-5", Fyfe *et al.* concluded. Their work involved the use of ^{29}Si and ^{27}Al NMR spectroscopy.

The speculation is that Union Carbide made the preemptive strike of suing Mobil after receiving an order for silicalite. The threat of a patent infringement suit would have to be removed from Union Carbide and the purchaser before a sale could go through. Union Carbide refuses to say whether an order has been received or whether any silicalite has been sold.

Stephen Budiansky

CORRESPONDENCE

This space was occupied by a letter withdrawn after the press deadline (see page 302)

South African ban

SIR — I do not wish to enter the debate about British participation in astronomy in South Africa but I must object to some statements in the letter from Dr David Evans (*Nature* 11 November, p.102).

He states that boycotts of the type proposed

are usually ineffective. The only boycott properly applied to South Africa has been in the field of sport, and this has been most effective, leading to integration in most branches. Many of us believe that the same kind of pressure applied to academic circles would achieve a similar result with beneficial consequences for the education of non-whites in that country. An academic boycott might be a blow to some sections of the community (almost all white) but would be welcomed by many others (almost all black) who derive great moral encouragement from manifestations of external sympathy with their cause.

He is of course right when he says the South African government has a problem of "terrorist activity", although this is a term one prefers not to apply to people who are simply trying to achieve equal rights for themselves. It must of course be evident that South Africa has brought this activity on itself; the problem would not have existed had it not chosen to suppress the majority of its people by refusing to provide ordinary democratic rights. Dr Evans is most certainly a victim of South African propaganda when he refers to the activists as being "inspired by the Soviet Union". Has he learned nothing at all from events in Zimbabwe? African people want independence and self-determination, not replacement of one dominant force by another.

Finally, he may believe that the best hope for a peaceable solution lies in an increase of cultural links from abroad. He must know that South Africa used to enjoy these links fully as part of the British Commonwealth and a respected member of the international community. It chose to isolate itself by inflicting a policy of apartheid on most of its inhabitants. Why should increased contact

with the rest of the world achieve more now than it did 35 years ago?
R. HOFFENBERG
*University of Birmingham,
Birmingham, UK*

Naming enkephalins

SIR — It is easy, as Morley (*Neuropeptides* 1, 231–235; 1981) has pointed out, to be misleading in attempting to specify the structures of enkephalins and their analogues. The commonest confusion may be caused if [Met]enkephalin, for example, is written as Met-enkephalin. This is because IUPAC-IUB recommendations, used throughout peptide chemistry, specify square brackets for substitutions and forms such as "Met-" for N-terminal extensions. Thus the form "Met-enkephalin" might well be necessary for extension of the basic sequence Tyr-Gly-Gly-Phe-Xaa to Met-Tyr-Gly-Gly-Phe-Xaa. The form [Met]enkephalin seems convenient as a simplification of the strictly systematic [Met]²enkephalin, which should be used in contexts where ambiguity is conceivable.

The recommendations for C-terminal extension and for partial sequences are illustrated by the identity of [Leu⁵]enkephalinyll-Arg-Arg with dynorphin(1–7) peptide.

References to the many printings of the 1966 IUPAC-IUB recommendations are given by Morley. They are now under revision, but there is no proposal to change appreciably any of the methods of designating N- and C-terminal extension of peptides, substitutions, deletions and partial sequences. The new recommendations are likely to be published in 1983.

H.B.F. DIXON

*Nomenclature Committee of IUB,
Cambridge, UK*

Chinese seismometry revisited

SIR — I write with reference to an article on a Chinese seismometer featured in "*Nature* 100 years ago" (ref.1). You may be interested to learn that the original article carried some errors. According to Joseph Needham¹, "Choko" was the Japanese version of the name of Chang Hêng (AD 78–139), a noted astronomer, and his seismometer dates from AD 132 rather than AD 136. Several reconstructive drawings are illustrated in

Needham's book and in 1910 another illustration was presented to this museum by the Japanese Imperial Department of Education.

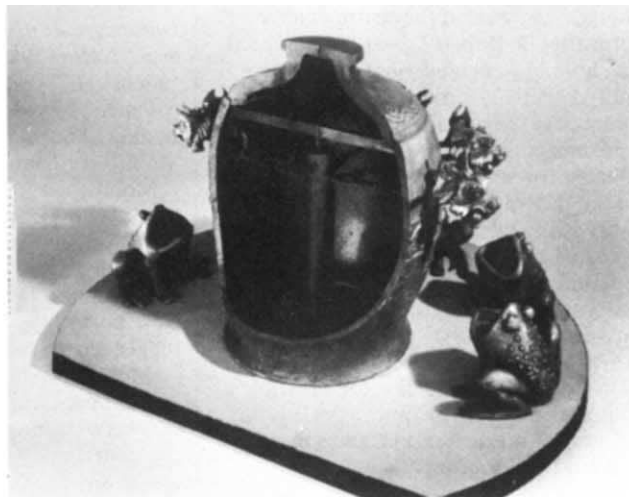
We currently exhibit a sectioned reconstruction of the seismometer (see below), given by the BBC, which has been demonstrated on television. In 1980 the Chinese Seismological Association presented a small statue of Chang Hêng to the Science

Museum; this too is on exhibition. The seismometer is also figured on one of a set of Chinese postage stamps, on display alongside Chang Hêng and his seismometer in our Geophysics Gallery.

ANITA MCCONNELL
(Curator)

*Science Museum,
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1. *Nature* 299, 779 (1982).
2. Needham, J. *Science and Civilization in China* Vol. 3, 626 (1959).



Science Museum

NEWS AND VIEWS

What future for the chaos concept?

Is classical chaos a kind of premonition of quantum uncertainty or something separately?

Now it may be possible to tell by means of an experiment, not endless argument.

What is chaos? During the past few years, physicists (not to mention philosophers of science) have been much preoccupied by the recognition — “discovery” would be the wrong word — that there are many macroscopic systems whose future behaviour cannot be accurately predicted. The old saw is that fourteen-day weather forecasting is impractical because, over such a time-scale, disturbances initially as negligible as the flapping of a single seagull’s wings may turn out to have a worldwide effect. In meteorology, the problem is to know which seagull’s flapping wings matter. There are two opposing answers to this absorbing question. Some will hold that the difficulty of calculating the effect of a pair of flapping seagull’s wings is temporary; better mathematics, or larger computers, will make it possible to tell whether a seagull’s wings have a greater effect when the Sun is at its zenith than at night, or whether mid-latitudes are more sensitive to disturbance than either tropical or polar latitudes. Others, however, take a different view. There are so many seagulls, so randomly distributed over the surface of the Earth but so constantly flapping their wings, that even if the mathematics of the meteorological problem were exactly soluble, the initial conditions would be intrinsically unknowable and the time-course of the development of the macroscopic system therefore intrinsically indeterminate. In this strictly classical sense, physical chaos is a way of referring to circumstances in which applied mathematics is imperfect — either the differential equations cannot be solved with sufficient accuracy or they (or the boundary conditions that should apply) cannot be accurately defined.

Inevitably, such circumstances are reminiscent of what happens in quantum mechanics: indeterminacy abounds. But to suppose that the similarity is meaningful would be mistaken. Quantum chaos is, after all, well-defined. You can measure the position of a particle but then not its momentum, or its momentum but then not its position, or some mixture of the two with uncertainties determined (apart from numerical constants) only by the mass of the object and Planck’s constant. Chaotic macroscopic physics is distinct from that line of experiment (or *Gedanken*-experiment) but not unconnected with the reason why the generalizations of thermodynamics were fashionable in the nineteenth century, before people pretended to be able to solve complicated differential equations exactly. That, surely, is how sober physicists would argue.

But if quantal and classical chaos are independent phenomena, should it not be possible to find evidence of some kind of analogue of classical chaos in the behaviour of quantum systems? This is the question taken up by R. V. Jensen of Yale University in one of this month’s issues of *Physical Review Letters* (49, 1365). Such problems are potentially important, as can easily be told by asking what will happen in the course of time to a large polyatomic molecule whose total vibrational energy is, say, either just above or just below the energy necessary to break one of the covalent bonds that hold the vibrating structure together. If the threshold is not quite surpassed, on classical physics the molecule will never shake itself apart. In a quantum framework, however, there will be a finite chance that the molecule will tunnel through from an entirely cohesive state to one in which there are two separate pieces. If the total vibrational energy is just greater than the threshold, quantum mechanics has nothing fresh to say — the rate of transition from one state to the other is greater, but the chance of sliding over a shallow barrier is not very different from that of tunnelling through it. On the face of things, only the case of a

classical polyatomic molecule whose energy is just above the binding energy of its weakest bond is interesting; then, on the face of things, it can only be a matter of time, perhaps a long time, before the molecule falls apart. The argument about the classical problem of chaos is essentially that of whether the equations of motion or the initial conditions can ever be sufficiently well-defined as to make the calculations of a single molecule’s fate determined. Most people would settle for an ensemble of molecules and some form of thermodynamics, statistical or otherwise.

Jensen’s question is different — that of whether a quantum system can be expected to behave with a degree of unpredictability transcending what Heisenberg uncertainty would require. In principle, such questions could be resolved by experiment. How frequently does a polyatomic molecule whose Hamiltonian is well-defined choose to shake itself apart? How easily can an electron be detached from a polyelectronic atom? Unfortunately, such systems, in which the quantum numbers are necessarily large, are complicated by the inherent quantal uncertainty. What Jensen has done is to devise a hypothetical experiment in which it should be possible to search for quantum chaos without the interference of quantum uncertainty in Heisenberg’s sense. Moreover, Jensen even promises that his experiment will be carried out.

The system considered is unusual, to say the least, and consists of the free electrons that must be supposed to exist above the surface of liquid helium. Such an electron is attracted to the surface by its electrostatic image charge, but prevented from penetrating the surface by the Pauli exclusion principle. Accordingly, in classical language, the electron oscillates between the surface and a distance (say a nanometre) determined by its kinetic energy. In quantum language, however, the electron is bound loosely in a potential well and, like an electron in any other kind of box, has a series of stationary states whose energy levels can be calculated by the manipulation of Schrödinger’s equation. (Technically, the problem is not as simple as it sounds, for the potential energy is inversely proportional to the distance from the surface, as with any other electrostatic charge attracted by its image.) Jensen solves the problem by transformation of the dynamical coordinates to action-angle variables; those with an algebraic interest will discover that the Hamiltonian (total energy expressed as momentum position) can validly be written in terms of a pair of other variables one of which is the square root of the maximum amplitude of the electron’s excursion from the surface, in which case the corresponding Hamiltonian variable is an angle.

The upshot is intriguing. Jensen supposes that electrons bound in this way near the surface of liquid helium are also perturbed by microwave radiation (whose wavelength is necessarily greater than the maximum excursion of the electrons and whose spatial variation can therefore be neglected). In classical language, electrons bound like this should be shaken free by the processes that lead to classical chaos. The interesting question is whether classical chaos persists in the quantum regime or whether it merges with it. The recipe that follows from Jensen’s work is to point a beam of microwave radiation at a liquid helium surface and to observe the rate at which electrons are shaken loose, or microwave power absorbed. The phenomenon should be evident at about 320 GHz if the measured dielectric properties of liquid helium are applicable. Rarely can a point in the philosophy of physics have been reduced to such a simple measurement.

Mantle methane — fool's gold?

from the Planetary Sciences Unit, University of Cambridge

THE controversial idea perpetrated by Professor Tommy Gold of Cornell University that hydrocarbons have their origin in the outgassing of methane from the Earth were the subject of a recent discussion meeting* at the Geological Society. Gold believes that the Earth contains in its interior a vast reservoir of primitive methane (CH_4) incorporated at the time when the solid body first formed. He suggests that over geological time, higher-molecular-weight hydrocarbons might be formed from a gradual leak of this primordial gas — non-biological petroleum appears from gas on its way out of the Earth's interior rather than being formed from increasing maturation of biological debris on the way down from the surface.

Gold's ideas have provoked a lively debate in the scientific community (speakers against included G. Eglinton, University of Bristol; J. Watson, Imperial College, London; and M. Schoell, Hannover) and, not surprisingly, have generated interest within the oil and gas industries. Although a general air of scepticism among both academics and commercial companies prevails, convincing evidence to negate the idea completely does not seem to be forthcoming. Since it is Gold's habit to dismiss arguments against as depicting special circumstances, the often heated exchanges continue. The Natural Environment Research Council has opted to maintain an open mind and a committee has been formed to consider deep-source gas under the chairmanship of Sir Peter Kent, who, in his policy statement to the meeting, pointed out that the wide range of geological circumstances in the UK provides a very suitable platform for appropriate investigations.

Gold proposes that methane, which is stable at high confining pressures and low oxygen fugacity, rises to the surface, not in a continual stream, but through a series of isolated traps. When the pressure in a particular enclosure exceeds a certain value, some of the gas is discharged into the next higher level by a momentary opening of the interconnecting pore spaces. Depending on the pressure-temperature-oxygen fugacity relationships experienced by the ascending gas, it will either reach the surface as methane (which may even spontaneously ignite if large volumes escape into the atmosphere) or carbon dioxide.

Gold's theory gains support from several independent lines of evidence. First, methane has been found outgassing from

mid-ocean ridge zones, crustal rifts, volcanoes and other areas of tectonic activity and also from a Russian deep drill-hole. The presence of methane at certain mid-ocean ridges correlates with that of ^3He (J. Welham and H. Craig *Geophys. Res. Lett.* 6, 829; 1979) which is universally accepted as a primordial telluric gas component incorporated at the time of the Earth's formation. The inference therefore is that methane is primordial in origin, exists deep within the mantle and perhaps even acts as the 'carrier' of the ^3He .

Second, he argues that various $\text{He}-\text{CH}_4-\text{N}_2$ relationships across some oil and gas fields cannot be explained unless two pre-existing, two-component reservoirs are mixed together in a deep location.

Third, at certain localities, Gold envisages that methane outgassing through the crust, is converted to CO_2 by methylotrophic bacteria and is then incorporated into carbonate cements. These deposits have unusually light carbon isotopic composition (-35 to -25 ‰), thereby making them distinct from normal carbonate sediments. The methane from which they are produced shows a depth-dependent increase in $\delta^{13}\text{C}$ (from -80 to -40 ‰) which can be explained by the continual removal of relatively heavy carbon to form carbonate as the methane rises to the surface. (All isotope measurements herein are relative and defined according to the δ convention, where

$$\delta^{13}\text{C} (\text{‰}) = \left[\frac{(^{13}\text{C}/^{12}\text{C})_{\text{sample}}}{(^{13}\text{C}/^{12}\text{C})_{\text{std}}} - 1 \right] \times 1000.$$

Thus, a single methane source is invoked to interpret the data. Opponents of the idea allude to the possibility of two methane origins to explain the trend — a deep thermogenically produced gas from a sedimentary source and a near-surface methane resulting from bacterial processes. Gold maintains, however, that the quantities of methane needed to produce some of the known carbonate deposits (for example, Cement, Oklahoma) would be vast (enough to fuel America at its current rate of consumption for 1,000 years) and therefore cannot have been generated by bacteria or cracking of higher hydrocarbons. If one accepts the Gold hypothesis that primordial methane alone is responsible for carbonate generation, then it is extremely interesting to note that in all cases the deposits lie directly above oil reserves. (Gold considers this as 'smoking gun' evidence that non-biogenic methane contributes to hydrocarbon formation.) However, even though only five per cent of all organic material produced in the geological

environment (G. Eglinton) can be proved by specificity of carbon skeleton to have a biological origin, the idea that the remaining material has been produced non-biogenically has still not found favour.

It is by no means universally accepted that the carbonaceous gases escaping from the Earth's surface are primordial in origin. The alternative view is that they are from recycled material — unfortunately the residence time for carbon in this recycling event is estimated to be greater than 10^8 years (M. Javoy *et al. Abstr. 5th int. Conf. on Geochronology, Cosmochronology and Isotope Geology*, p.173, Japan, 27 June–2 July 1982) and so no useful information would be yielded by ^{14}C measurements. However, the stable isotope composition of methane (both D/H and $^{13}\text{C}/^{12}\text{C}$ determinations) may help to resolve the issue. Schoell certainly argued convincingly that the isotope systematics of methane in Lake Kivu cited by Gold as correlating with ^3He could be accounted for by reduction of magmatic carbon dioxide together with gas derived by fermentation.

It may be ironic that Gold believes that some of his strongest evidence for a primordial origin (as above) derives from stable isotope data, given the paucity of relevant information. Traditionally, the primordial $\delta^{13}\text{C}$ value of the Earth is quoted as being between -5 and -7 ‰. However, diamonds, which are undoubtedly of deep-seated origin and might be expected to have once resided close to the primordial carbon reservoir, display a range in $\delta^{13}\text{C}$ values from 0 to -30 ‰ (D. Deines *Geochim. cosmochim. Acta* 44, 943; 1980) for reasons as yet unknown. Furthermore, the $\delta^{13}\text{C}$ value of ^3He -correlated methane outgassing from mid-ocean ridges is found to be about -18 ‰ (J. Welham *EOS* 61, 996; 1980). How these data relate to $\delta^{13}\text{C}$ values from the other major carbon reservoirs [-7 , -27 , -41 ‰ for atmospheric CO_2 , CO and CH_4 , respectively, or about 0 ‰ for most carbonate deposits, around -25 ‰ for organic matter (-35 ‰ in the Precambrian) and around -25 ‰ for the reduced carbon in magmatic rocks] remains an enigma mainly because of the small numbers of analyses of total carbon and gases in critical samples ($\delta^{13}\text{C}$ measurements are needed from deep-seated rocks, diamonds and kimberlites, fluid inclusions and locations such as basement fractures, kratons, subduction zones, back-arcs, fore-arcs, transform faults and so on).

Methane is a stable form of carbon in the early solar nebula and therefore should have contributed to the primitive Earth. Unlike the primordial noble gases, most of

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* The discussion meeting 'Fossil fuel: Biogenic versus non-biogenic' was organized by the Petroleum Geochemistry Group of the Geological Society and held at Burlington House, London, on 7 October 1982.

which are thought to have been lost early in the Earth's history, the other volatiles (H_2O , carbon-containing compounds and so on) were largely retained. Whether one likes Gold's hypotheses or not, and the man confessed to being 'as slippery as oil', he left his audience with many unresolved

issues. The possibility that primordial methane still outgasses from the Earth, forms commercially exploitable accumulations and is involved with higher hydrocarbon formation cannot be dismissed out of hand; constructive research and exploration is necessary. □

An enlarged view of intracortical connectivity

from Nicholas V. Swindale

THE conventional view of intracortical connectivity, derived from the results of Golgi¹ and degeneration studies², is that lateral connections between nerve cells in the cortex are confined to distances of 1–2 mm or less. However, recent work involving studies of the transport of the enzyme horseradish peroxidase (HRP) following either extracellular^{3–5} or intracellular^{6,7} injection into the cortex suggests that some intracortical connections may extend over much larger distances. The functional significance of these long-range connections is unclear, but they do seem to be related to some aspect of columnar organization since, like many of the inputs to the visual cortex from other cortical and sub-cortical areas, they are organized into regularly spaced patches with a periodicity of about 1 mm or less.

The clearest evidence comes from some experiments of Gilbert and Wiesel^{6,7} in which they recorded intracellularly from neurones in the visual cortex of the cat, and then filled them with HRP so that their axonal and dendritic morphology could be reconstructed. Many pyramidal cells were found to have axons that extended laterally over distances of up to 3–4 mm, making periodically spaced clusters of connections (see the figure). The clusters of any one cell may be present in more than one cortical layer, and when they are, they are often in register, suggesting a relationship to some aspects of columnar organization. One reason why this observation has not been made before is that the intracortical axons are often myelinated, and thus fail to impregnate with the Golgi stain; another is that the patchy distribution of axon terminals is often only clearly evident when three-dimensional computer graphics techniques are used to rotate the reconstruction of an axon so that it can be viewed from a particular direction.

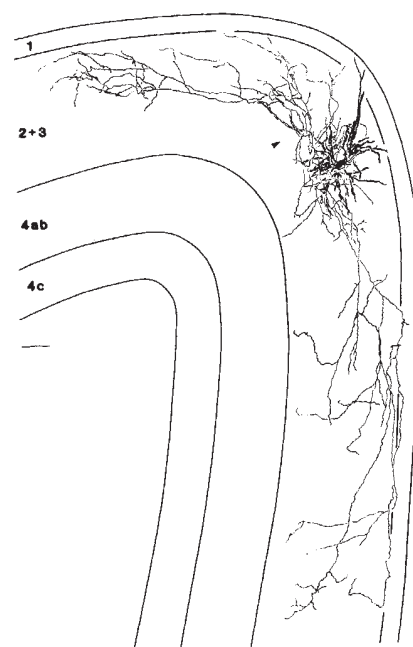
Another demonstration of what seems to be the same phenomenon comes from some

experiments performed by Rockland and Lund^{3–5} in which small extracellular injections of HRP were made into the visual cortex of tree shrews and monkeys. The HRP is transported into periodically spaced patches of cells and axons extending for distances of several millimetres in the upper layers of the cortex surrounding the injection site. Interpreting these results in terms of connections between different columns poses something of a problem however. Suppose that the long-range connections only link up columns of the same type — that, for example, columns of vertically orientation-selective cells only make connections with other such columns, and that columns for horizontal and oblique orientations are similarly interconnected. An HRP injection that was small enough to label the projections and inputs of only one such column would show the observed patchy distribution of HRP transport to similar neighbouring columns. But Rockland and Lund believe that their injections should have been big enough to label the connections with not just one such column, but many of them. As different columns occupy complementary regions of tissue, the pattern of HRP transport expected, on this line of reasoning, should have been much more uniform than was observed. Rockland and Lund's interpretation of the patchy HRP transport therefore is that the long-range connections link up only some regions of cortex and not others — something they refer to as a fixed-position connective lattice.

Other explanations of Rockland and Lund's result cannot be ruled out at this stage however. One possible complication is that the HRP injection sites might be much smaller than they seem. Much more HRP might be taken up from the very small region of damage caused by the injection than from intact cells. Another possibility is that some cells are intrinsically more likely to take up HRP than others, and that these cells have a patchy distribution. This might conceivably be related to differences in transmitter type, or metabolic activity, for which there is evidence of a patchy variation in the monkey^{8,9}.

Even if these complications can be ruled out, patchy local uptake of HRP from a

large injection does not necessarily imply that some regions of cortex possess long-range connections while others do not. Mitchison and Crick¹⁰ have recently proposed some simple and plausible rules of connectivity between orientation columns that could explain the Rockland and Lund result. They suggest that connections between columns are not distributed radially, but are oriented in a direction that is related to the orientation selectivity of the cells that are being connected. As an example of why this might be so, consider Hubel and Wiesel's proposal¹¹ that complex-cell receptive fields are made by connecting together a number of simple-cell receptive fields. As a complex cell will respond to a line anywhere in a roughly circular receptive field, and the simple-cell receptive field is elongated, it would be necessary to join together a row of simple cells, the row running perpendicularly to the receptive field orientation. In the cortex therefore, the connections between columns would have to be made in a direction at right angles to the orientation preference of the cells in the columns, mapped onto the cortical surface. In the case where the columns form parallel stripes running in a horizontal direction in the visual field (as they do in both cats and tree shrews), vertically selective cells would have axonal fields elongated in the same direction as the stripes, while horizontally selective cells would have axons cutting across the stripes and making patchy connections. Given this pattern of connectivity, one can predict the local distribution of HRP uptake from an injection large enough to label the pro-



A pyramidal cell in layer 2 + 3 of the visual cortex of the cat traced by intracellular injection of horseradish peroxidase. Some axons extend laterally over distances of up to 3–4 mm.

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jections to and from many adjacent columns. It is perhaps not intuitively obvious what this would be, but computer simulations show that the distribution of label would resemble that observed by Rockland and Lund, the patches having the same spacing as the orientation columns and running in a similar direction.

Mitchison and Crick's hypothesis of course applies to any system of long-range intracortical projections whose direction in the cortex bears some consistent relation to the orientation preference of the cells being connected (for example, to a system of connections designed to produce very elongated receptive fields by joining them end to end rather than sideways on). In this broad sense the hypothesis seems, *a priori*, more likely to be correct than not. Gilbert and Wiesel's anatomical results do provide some support for the idea, since they show that the axonal fields of cortical neurones are often elongated, though exactly how the direction of elongation relates to the receptive field orientation is not yet quite clear.

At this stage it might be better to wait for more experimental results before speculating further on the nature and

function of the patchy intracortical connections. One needs to know, for example, how the HRP patches relate to other columnar systems, such as those for orientation and ocular dominance, and whether the patches formed by one cell innervate columns with similar, or different properties from those of the cell itself. It should also be a fascinating exercise to discover whether the patchy intracortical connections follow the same rules that characterize the development of the geniculocortical projection, and whether visual experience can similarly alter their morphology. □

1. Lorente de No, R. in *Physiology of the Nervous System* (ed. Fulton, J.F.) (Oxford University Press, New York, 1938).
2. Fiskens, R.A., Garey, L.J. & Powell, T.P.S. *Phil. Trans. R. Soc. B272* (1975).
3. Rockland, K.S. & Lund, J.S. *Science* **215**, 1532 (1982).
4. Rockland, K.S., Lund, J.S. & Humphrey, A.L. *comp. Neurol.* **209**, 41 (1982).
5. Rockland, K.S. & Lund, J.S. *Soc. Neurosci. Abstr.* **8** (in the press).
6. Gilbert, C.D. & Wiesel, T.N. *Nature* **280**, 120 (1979).
7. Gilbert, C.D. & Wiesel, T.N. *J. Neurosci.* (in the press).
8. Hendrickson, A.E., Hunt, S.P. & Wu, J.-Y. *Nature* **292**, 605 (1981).
9. Horton, J.C. & Hubel, D.H. *Nature* **292**, 762 (1981).
10. Mitchison, G. & Crick, F.H.C. *Proc. natn. Acad. Sci. U.S.A.* **79**, 3666 (1982).
11. Hubel, D.H. & Wiesel, T.N. *J. Physiol., Lond.* **160**, 106 (1962).

174; 1979). One of these might weigh as much as 10^{16} protons and, hence, be too heavy for production by particle collisions except in the first 10^{-35} seconds of the life of the Universe, if the latter was indeed created in a big bang as is currently believed. These monopoles would be expected to survive unscathed until the present day but their signature was not observed before this year.

Several suggestions were made to reconcile the discrepancy between Cabrera's upper limit and the Parker bound. One was that the monopole was as heavy as the Planck mass — 10^3 times heavier than previously assumed — thereby increasing the Parker limit. Another was that the distribution of monopoles is not uniform in the Universe, clustering, for example, in the galactic haloes near where we reside or near massive objects such as our Sun.

The experimental challenge was seen to be the detection of a monopole flux as low as the Parker figure. Coincidence experiments using superconducting coils in 10^{-8} G with area 10^2 cm² and in 10^{-2} – 1 G with area 10^2 – 10^4 cm² were described. These detectors are sensitive to the passage of a magnetic charge, whatever its velocity. Large-area (10^6 cm²) scintillators and Cherenkov counters, such as those used in proton decay experiments, are being adapted, and a 'football field' size (10^8 cm²) acoustic detector is being planned. Yet another approach attempts to discover monopoles trapped in matter by positioning a superconducting coil under an iron ore plant conveyor belt, thereby monitoring more than a million tons of ore per year.

Due to the effect of the galactic magnetic field the velocity of a free monopole is expected to be less than about 10^{-3} of the speed of light, which is somewhat less than the speed of an electron in an atom. This limits the type of energy-loss mechanisms available to the passing monopole, and the optimization of detector materials depends on a better understanding of the effect of these slow monopoles. The detectors based on superconducting coils neatly avoid these difficulties.

Physics thrives on paradoxes created by interactions between different ideas, in this case the commonly accepted pictures of cosmology and particle physics. Hence, one theoretical challenge was the construction of a combined cosmology and particle physics theory which did not overproduce monopoles. It seemed that an inflationary universe is an essential ingredient at least at very early times (J.D. Brown & M.S. Turner *Nature* **298**, 801; 1982).

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Catch a passing monopole

from John Ficenec and David Olive

THE first international workshop to focus exclusively on both the theoretical implications and experimental techniques relevant to the detection of magnetic monopoles (isolated magnetic charges) was held recently at Wingspread*. More than 80 experts from an enormous spectrum of physics subdisciplines were brought together in Frank Lloyd Wright's last and largest prairie home through the courtesy of the Johnson Foundation. The meeting was called in response to an observation this spring by Blas Cabrera (*Phys. Rev. Lett.* **48**, 1378; 1982) of an event whose interpretation was consistent with the passage of a magnetic monopole.

The observation has not been corroborated either by Cabrera or others. The flux limit placed on the monopole after collecting more data and a refined analysis was 10^{-10} monopoles cm⁻² sr⁻¹ s⁻¹. This flux remains uncomfortably high when compared with the so-called Parker bound (E.N. Parker *Astrophys. J.* **160**, 383; 1970) of 10^{-15} monopoles cm⁻¹ sr⁻¹ s⁻¹ at which galactic monopoles would dissipate the observed magnetic field of the Galaxy faster than it could be regenerated. Theoretical scenarios to resolve this

discrepancy and to explain the candidate event were presented and debated. Numerous experiments, many in the development stage, were discussed. The topic remained in a state of flux with numerous theoretical and experimental questions unanswered, but the participants were optimistic that many problems could be resolved within the next few years.

One of the aims of physics is unification — the organization of the forces of nature, nuclear/strong, electromagnetic, weak and perhaps even gravitational, as different facets of a single symmetrical entity. Calculations indicate that the symmetry is only apparent at energies of 10^{14} GeV, and the characteristic differences between forces at ordinary energies arise because of symmetry breaking. Although this theory is elegant, the enormous disparity in scale between 10^{14} GeV and the proton rest mass energy of 1 GeV is unsettling, particularly since the high-energy regime is inaccessible to any conceivable particle accelerator. However, one consequence is that protons will decay (see *News & Views* **299**, 295; 1982) with a lifetime of $10^{31\pm2}$ yr which should be observable in large-volume experiments already set up and running.

Another prediction of the grand unified theories is the existence of magnetic monopoles which elementary text books tell us do not exist (see *News & Views* **277**,

*The 'Magnetic Monopole Workshop' held at Wingspread near Racine, Wisconsin, on 14–17 October was sponsored by the North Atlantic Treaty Organization, the US Department of Energy, the US National Science Foundation and the Johnson Foundation. The proceedings will be published in early 1983 by Plenum, New York.

Another theoretical challenge was the elucidation of a new effect which could change the previous estimates for proton decay if monopoles were abundant. It has been known since the last century that the relative angular momentum of an electric and a magnetic charge has a term equal to the product of their charge times the unit vector pointing along the line joining them. If the projectile passes straight through the

target in a collision, then conservation of angular momentum means that the sign of electric charge must reverse in order to balance the reversal of the unit vector. This means the monopole must absorb two units of charge. In a theory including the strong interaction gauge group, baryon number could also be absorbed by the monopole, thereby catalysing proton decay to give a distinctive experimental signature. □

Recent (1981–82) evolution of galactic haloes

from Virginia Trimble

THE halo of our Galaxy is perhaps a bit older, a bit richer in heavy elements, a bit less massive and undoubtedly a bit more complicated than it was before the recent meeting* of the International Astronomical Union in Greece.

A typical spiral galaxy, whose most conspicuous component is a flat, rotating, armed disk, also has a halo of stars which includes the oldest stars in the galaxy, and which bears at least a passing resemblance to the other major class of galaxy, the ellipticals. Many halo stars are still gravitationally bound in globular clusters of 10^5 – 10^6 stars in which they formed. There is also a 'scattered field' star population, generally advertized as consisting of fugitives from the clusters.

Among the things we would like to know about the haloes of our own and other galaxies are (1) the ages of the oldest stars (because this sets a significant lower limit to the age of the Universe), (2) their chemical compositions and how they acquired their heavy elements (since, after all, stars formed out of virgin big bang material should be pure hydrogen and helium, and we see no stars like that), (3) whether the globular cluster and field stars are really part of a single population, (4) whether the dynamical structure of haloes is similar enough to that of elliptical galaxies to suggest a common origin (this would considerably simplify the difficult problem of making galaxies out of lumps in the early Universe) and (5) whether there is anything in the haloes besides the stars we see, and, if so, how much of what (because significant mass there will affect our estimate of the average density of the Universe and so its future evolution). New evidence bearing on these questions appeared at the meeting and in recently published papers.

How old are the stars? Rather older than is entirely convenient! The standard method of measuring the age of a star cluster is to compare the positions of the stars in a graph of brightness against surface temperature or colour (HR

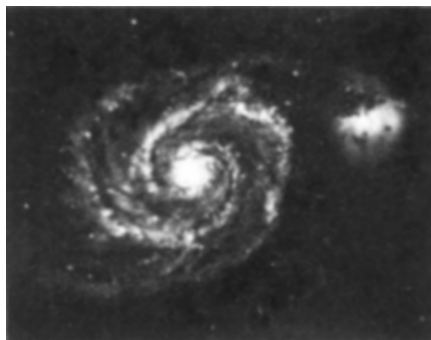
diagram) with calculated versions of what those positions ought to be as a function of the age of the cluster stars (and, unfortunately, their chemical composition and other variables also). The integrated brightness and colour of a cluster can be used somewhat similarly. These methods have yielded values of 7 – 25×10^9 yr in the past. R. Cannon (Royal Observatory, Edinburgh) and B. Carney (University of North Carolina) reviewed eight more-or-less independent recent applications that concur on ages of 15 – 18×10^9 yr, at least for the metal-poor clusters. If stars this old are to be fit into a standard Friedmann model of the expanding Universe, then the Hubble constant, H_0 , must be less than about $65 \text{ km s}^{-1} \text{ Mpc}^{-1}$; and if H_0 is to be ≥ 50 , then the Universe must be a low-density (ever-expanding) one as well. According to Carney, the likely errors go in the direction of making these ages, if anything, underestimates. On a related topic, S. Faber (Lick Observatory) discussed evidence from UV photometry of elliptical galaxies indicating that star formation in them also stopped some billions of years ago (though not necessarily so many as 15).

What is the chemical composition of the halo stars? It varies. There has been agreement for some years that the more metal-poor of the globular clusters have

about 1 per cent of the solar heavy-element abundance. At the other end of the scale, there has been a continuing discrepancy between photometric studies, which found the most metal-rich clusters to come within a factor of three or so of solar abundance (like the oldest disk stars), and spectroscopic studies, which found much lower abundances. G. Gustafsson (University of Uppsala) drew attention to several sources of uncertainty in both methods, and reported spectroscopic work by M. Bessell (Mt Stromlo Observatory) that yields abundances close to the photometric (higher) values. If this is the right answer, then evidently chemical processing (birth and death of stars) continued in the halo until the remaining gas collapsed down into a disk and began forming stars there. This is rather easier to understand than a picture in which there is a large gap in composition between the youngest halo stars and the oldest disk ones.

Is there a single halo population? Apparently not. The field and globular cluster stars seem to have the same dynamical characteristics, ages and range of metal abundances, but there are detailed differences. R. Kraft (Lick Observatory) suggested that the field stars have, on average, smaller amounts of nitrogen, larger amounts of carbon and a narrower range of abundances of both than do cluster members. This implies either that the field stars are less highly evolved (less thoroughly mixed), meaning that they are younger or less rapidly rotating or something, or that the cluster members have been more thoroughly contaminated by heavy elements made in supernovae in the clusters themselves. Differences between field and cluster stars in the ratio of barium to iron¹ at least weakly support the first, evolutionary, sort of hypothesis. A difference between cluster and field stars in the maximum metal abundance to be found among RR Lyrae and type II Cepheid (pulsating variable) stars is not easily explained by either mechanism². Thus, if the field stars have merely escaped from the clusters, they have not done so at random.

Are elliptical galaxies and the spheroidal parts of spirals the same sort of thing? Again, apparently not. The general shape and ranges of compositions and ages are similar, but there are significant differences. Spiral bulges (the central part of the spheroidal component) have fewer globular clusters per unit luminosity than do ellipticals³. This could only be fit into a unified model if clusters were somehow torn apart by the process of disk formation, which seems unlikely. In addition, ellipticals typically show a steeper gradient of composition, from metal-rich at



A typical spiral galaxy contains two basic components: a flat, rotating armed disk and a spherical halo of stars which, at their most concentrated, form the central bulge of the disk.

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*The meeting was held in Patras, Greece on 16–26 August 1982.

the centre to metal-poor at the edges, than do spiral bulges⁴. This might just mean that chemical evolution operated longer or more efficiently in them. But, finally, well studied haloes show overall net rotation⁵, which is not found in most elliptical galaxies and could not very well have been introduced after galaxy formation was complete.

Finally, what, if anything, is in the haloes besides the stars we see? Probably, though not certainly, up to 90 per cent of the mass of the Universe. J. Ostriker (Princeton University) reviewed observational evidence of measured galactic masses that increase roughly linearly with the radius out to which the measurement is made, from the inner 10 kpc sampled by rotation curves, through the 20–100 kpc range sampled by orbits of globular clusters and satellite galaxies, out to the 1–3 Mpc sampled by dynamics of rich clusters (see ref. 6 for another recent summary).

The implication is that a typical large galaxy has associated with it about $10^{12} M_{\odot}$ (mass of the Sun) in some form, of which we can see only about 10 per cent in disk and halo stars. D. Schramm (University of Chicago) reviewed the arguments indicating that the invisible material is quite likely to be in the form of exotic particles such as non-zero-rest-mass neutrinos, photinos, or gravitinos. This evidence is largely negative — adequate amounts of ordinary matter could not have escaped detection, either directly or through their influence on big bang synthesis of helium and deuterium.

But two noteworthy arguments against extensive invisible mass, at least in some galaxies, surfaced at the meeting. First, J. Hunter (University of Florida; see also ref. 7) presented a rotation curve for the barred spiral NGC3992 showing $2 \times 10^{11} M_{\odot}$ within the inner 19.7 kpc (on the de Vaucouleurs 'short' distance scale) and velocities of satellite galaxies around 3,992 indicating at most $4 \times 10^{11} M_{\odot}$ within 59 kpc, so that $M(R)$ must increase much less steeply than linearly with R , and might stop increasing entirely beyond about 20 kpc. The most relevant response to this is that other galaxies with satellites do show rising $M(R)$, and that even H_I profiles (roughly, rotation curves) yield masses of order $10^{12} M_{\odot}$ for some galaxies, when the data extend out far enough, as witness the line widths near 800 km s⁻¹ for NGC5635 and IC4553 (ref.8).

Second, A. Kalnajs (Mt Stromlo Observatory) showed results of his calculations of the rotation curves

produced by various sorts of mass distributions. The result most impressive to his audience is that it is quite possible to get a flat rotation curve, in which velocity versus radius does not drop off at large radius, without invoking a massive, unseen halo component. The secret is a model in which halo and disk have the same constant ratio of mass to luminosity (3–7 in solar units for the galaxies he fit) out to the last measured point, where both end sharply, so that we cannot measure the rotation curve out where we would expect velocity to be dropping with increasing radius in a keplerian way. The four galaxies fit in this way have data extending out to 5, 8, 12 and 25 kpc from their centres; and the fits do

not much constrain what is going on outside that. The mass distributions may, indeed, end sharply as in the models or may continue out to 100 kpc or more without distorting the rotation curve near the centre. Which is right must be determined from data that probe further out, such as those from satellite galaxies, binary galaxies and rich clusters, invoked by Ostriker and others in support of massive haloes. Many participants nevertheless came away feeling that the evidence for massive haloes had largely disappeared. This is, perhaps, a natural over-reaction to having had the arguments in favour presented so vigorously at so many previous meetings! □

Human monoclonal antibodies

from Karol Sikora and A. Munro Neville

Now that the application of monoclonal antibodies to the diagnosis and treatment of human disease has become the major goal of many investigators, a limiting factor is the availability of human rather than mouse or rat monoclonals. Human monoclonal antibodies are particularly needed for administration to patients for diagnosis and therapy in order to minimize the problems of administration of a foreign animal protein, with the risks of anaphylaxis and the clinical manifestations of immune complex formation, as well as abrogation of the antibody's effects.

The short history of human monoclonal antibody production has been fraught with technical difficulties but several novel systems now hold out promise. Initial attempts used available mouse and rat myeloma lines for fusion^{1–3}. Unfortunately the resulting hybridomas preferentially lose human chromosomes, eventually eject the chromosomes coding for immunoglobulin production and thus lose the ability to produce monoclonal antibodies. Although early and repetitive cloning of the hybrids can reduce the shedding of human chromosomes, the development of more stable human-human hybridoma systems seems desirable.

Accordingly several groups have developed human systems from which hybridomas may be created. Unfortunately many human myelomas are difficult to grow in tissue culture and have low growth rates and poor immunoglobulin secretion. Nonetheless, certain lines have been 'adapted' to grow in culture in genetic selection conditions in which the growth of the myeloma is suppressed but the hybridomas continue to propagate. There have, however, been difficulties with mycoplasma infection in myeloma stocks. Indeed many investigators have been unable to obtain any hybridomas at all with

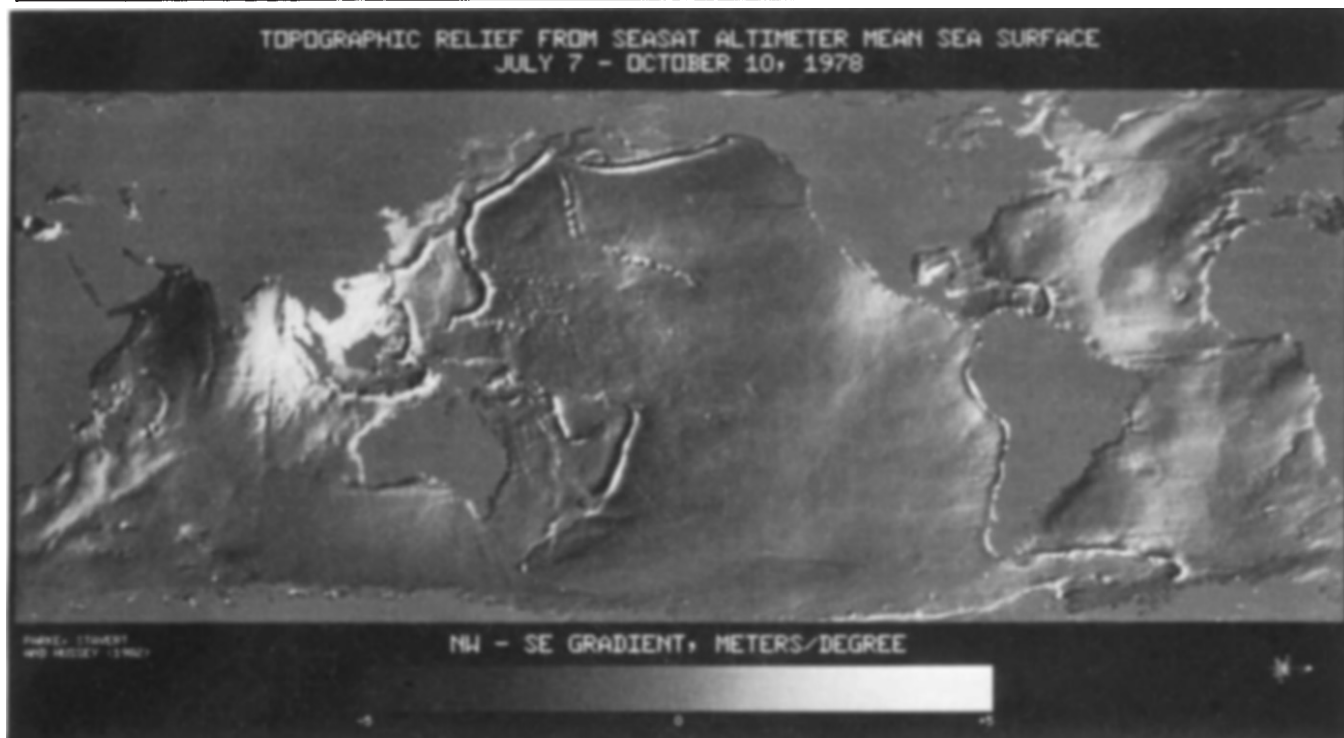
lines donated by certain laboratories, causing considerable controversy.

The use of several human myeloma or lymphoblastoid lines was discussed at a recent workshop in Cambridge held under the auspices of the Ludwig Institute for Cancer Research (LICR). The ideal properties of such a cell system include high fusion frequency, high cloning efficiency, the ability to grow rapidly in non-stringent serum conditions, no secretion of myeloma or lymphoid cell immunoglobulin, and yet the production of large amounts of immunoglobulin after fusion to suitable lymphocyte donors. M. O'Hare (LICR, London) described his experience with the cell line LICR-LON-HMy2 (ref.4) — a derivative of the human 'myeloma' ARH77 (ref.5). This lymphoblastoid line expressing Epstein-Barr virus (EBV) antigens appears to be the best available system. Reasonable fusion frequencies have been obtained from a variety of human lymphocytes but the immunoglobulin secretion rate is low at 0.2–8.0 µg ml⁻¹ in 24 h.

K. Sikora (LICR, Cambridge) reported that use of the same line. Intra-tumoral and regional node lymphocytes from the tumour-draining nodes of 180 patients resulted in the successful production of new immunoglobulin-producing hybrids in 24 cases⁶. When these were screened for antitumour antibody activity, weak binding was detected in only nine hybridoma supernatants. More success was achieved when fusing intra-tumoral lymphocytes from patients with malignant glioma, and three monoclonal antibodies to lung cancer were obtained by fusing lymphocytes from intra-thoracic lymph nodes removed at the time of surgical

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1. Spite, M. & Spite, F. *IAU Symp.* **80**, 169 (1978).
2. Carson, T.R. & Stothers, R. *Astrophys. J.* **259**, 740 (1982).
3. van den Bergh, S. *Pub. astr. Soc. Pac.* **94**, 495 (1982).
4. Griessmuth, D., Hyland, A.R. & Jones, T.J. *Astr. J.* **87**, 1106 (1982).
5. Huchra, J., Stauffer, J. & Van Speybroeck, L. *Astrophys. J. Lett.* **259**, L57 (1982).
6. Press, W. & Davis, M. *Astrophys. J.* **259**, 449 (1982).
7. Gottesman, S.T. & Hunter, J.G. Jr *Astrophys. J.* **260**, 65 (1982).
8. Mirabel, I.F. *Astrophys. J.* **260**, 75 (1982).



THE gravitational signature of tectonic features can be clearly seen in the topographical relief maps of the world's ocean surface shown above. Data from the Jet Propulsion Laboratory's Seasat oceanographic satellite were used to produce the map after processing to emphasize steep, small-scale features that tilt towards or away from the north-west.

The Mid-Atlantic Ridge and the trenches along the west and north-west margins of the Pacific Plate are clearly visible, as are the chain of Hawaiian seamounts.

The extent to which a seamount affects the local gravity field depends on the age of the underlying crust when the seamount was formed. If the seamount formed on young flexible crust, the crust deforms

around the seamount, compensating the effect of the seamount's extra mass. Seamounts formed on older, more rigid crust are not compensated in this way, resulting in a gravitational signature that is more apparent at the sea surface. For use of similar GEOS-3 altimetric data to investigate sea basin formation see *Nature* 299, 117; *News and Views* 299, 104; 1982. □

treatment⁶. Again, the secretion rate of patient-derived immunoglobulin was low. None was tumour type specific.

Coté (Sloan Kettering Institute, New York) described his work involving over 80 fusions with three different hybridoma systems — SK007 (which had been rendered free from mycoplasma infection⁷), LICR-LON-HMy2 and NS1, the standard mouse myeloma⁸. Immunoglobulin secretion rates varied from 1 to 100 $\mu\text{g ml}^{-1}$ with no consistent differences between the three fusion systems. Early cloning and stringent culture conditions seemed to be important prerequisites for success with inter-species hybrids if continued production of human immunoglobulin for long periods was to be achieved. Some human antibodies with interesting specificities to tumour cytoskeletal components were demonstrated by elegant immunohistological studies using human tumour lines. Using the lymphoid cell lines UC729-6, I. Royston (University of California, San Diego) was able to produce monoclonal antibodies which reacted with various cancer cell lines, but not normal lymphocytes, granulocytes, red cells or fibroblasts. These antibodies, although binding weakly to a variety of tumour cells, were in no way tumour specific. S. Clark (University of Glasgow) used diethyl pyrocarbonate⁹ in

concentrations near the mean lethal dose for myeloma cell lines as a selection mechanism for cells whose enzyme components were complemented after fusion. This allowed the outgrowth of hybridomas. He described work using the human myeloma RPMI 8226 and a sub-line of the EB4 myeloma — SJR22C. Again, there were problems of low fusion frequency and poor cloning efficiency.

Perhaps most interesting was D. Crawford's (Imperial Cancer Research Fund, London) account of making monoclonal anti-influenza and anti-rhesus D antibodies by infecting peripheral blood lymphocytes from donors with EVB. Several groups have tried this technique over the last few years without much success, the main stumbling block being the cloning of the EBV-transformed lymphoblast. Using a combination of *in vitro* stimulation by immunizing antigen

and early cloning by limiting dilution, Crawford was able to demonstrate that not only could useful antibodies be produced but also the concentration of immunoglobulin produced was at least equivalent to the best human hybridoma systems now available. The necessary ingredients in the recipe for success are still unclear. Several groups are investigating the possibility of initial EBV transformation, selection of cells with the required antibody specificity and subsequent immortalization by cell fusion in a hybridoma system.

We are still unfortunately plagued by technical problems in the production of human monoclonal antibodies, not least in discovering ways of collecting those lymphocytes that produce antibodies with the specificities being sought for different experimental purposes. It is too early to know whether all the current efforts will result in biologically interesting molecules with fundamental and clinical potential or just additional reagents with little difference from other available rodent monoclonal antibodies that can be obtained with significantly less effort. Nonetheless, the prospect of the former result will stimulate many groups to study and develop human hybridoma systems in the near future. The outcome could be exciting. □

1. Levy, R. & Dille, J. *Proc. natn. Acad. Sci. U.S.A.* 75, 2411 (1978).
2. Schlom, J., Wunderlich, D. & Teramoto, U. *Proc. natn. Acad. Sci. U.S.A.*, 77, 6841 (1980).
3. Sikora, K. & Wright, R. *Br. J. Cancer* 43, 696 (1981).
4. Edwards, P., Smith, C., Neville, A.M. & O'Hare, M.J. *Eur. J. Immun.* 12, 641 (1982).
5. Burk, K.H., Drewinko, B., Trujillo, J.M. & Ahearn, M.J. *Cancer Res.* 38, 2508 (1978).
6. Sikora, K., Alderson, T., Ellis, J., Phillips, J. & Watson, J. *Br. J. Cancer* (in the press).
7. Kaplan, H. & Olsson, L. *Proc. natn. Acad. Sci. U.S.A.* 77, 5429 (1980).
8. Kohler, G. & Milstein, C. *Nature* 256, 495 (1975).
9. Wright, W.E. *Exp. Cell Res.* 112, 395 (1978).

The recovery of Halley's Comet

from David W. Hughes

AMERICAN and European astronomers have been competing with each other to be the first to sight Halley's Comet. It was last seen at the end of May 1911, heading away from the Sun and passing below the orbit of Jupiter. It reached aphelion in 1948 and has been racing back towards the inner Solar System ever since.

The Americans have won. *International Astronomical Union Circular*, number 3737, reported that D. Jewitt, G. Danielson, J. Gunn, J. Westphal, D. Schneider, A. Dressler, M. Schmidt and B. Zimmerman recovered the comet on the night of 16 October 1982. They used a charge-coupled device at the prime focus of the 5.1 m (200 inches) Hale telescope at the Palomar Observatory, California. Charge-coupled devices are much more sensitive than photographic plates and with the 5.1 m telescope can detect objects fainter than 25th magnitude, some 50 times fainter than the photographic limit. Five 8-min exposures were taken through a broad-band blue filter. An object was detected near the expected position of the comet, moving in the expected direction at the expected velocity. It had a magnitude of 24.3 ± 0.2 (a change of unity in magnitude is equivalent to a factor of 2.5 in brightness, so Polaris the Pole Star is 7×10^8 times brighter than Halley's Comet at present). Confusion with an asteroid is regarded as extremely unlikely. The recovery was confirmed by M. Belton and H. Butcher who obtained accurate cometary coordinates on the nights of 18 and 20 October using a cryogenic camera and charge-coupled device on the 4 m Mayall telescope at the Kitt Peak National Observatory (*IAU Circ.* no. 3742). Their report of the unsuccessful search during winter 1981–82 can be found in *Nature* (298, 249; 1982).

No coma was detected, but this is hardly surprising because the surface temperature of the nucleus is expected to be about 120K. The recovery brightness indicates that the nucleus of the comet, assuming that it has a geometric albedo of 0.05, is about 9 ± 1 km across.

Halley's Comet, now termed 1982i, because it is the ninth new or recovered one this year, is in the constellation of Canis Minor. On 16.47569 October it was at a Right Ascension of 7 h 11 min 01.9 s and a declination of $+9^\circ 33' 03''$, putting it only 0.6 s west of the position predicted by D. Yeomans (*The Comet Halley Handbook*, JPL 400-91 1/81; 1981). The comet is early and will pass perihelion some 8.6 h before the expected time of 9.66128 February 1986. At recovery Halley's Comet was

11.04 AU away from the Sun, so it has broken two records — it is the most distant comet ever seen, beating Stearn's Comet (1927 IV) by about 1 AU, and the faintest comet ever recorded.

The fact that Halley has been recovered 3.3 yr before perihelion passage is also amazing. At its 1910 return it was first seen on 11 September 1909 about 7.5 months before perihelion by Max Wolf, who used a one-hour photographic exposure with the 72 cm Heidenberg reflecting telescope. It was then at the outer edge of the asteroid belt about 3.4 AU from the Sun and of magnitude 16. It was 10 arc min from the predicted position and the previously predicted perihelion passage time was 3.03 days too early.

The Europeans were also first at the

return before that. Dumouchel, the director of the Collegio Romano Observatory, obtained the first telescopic view of the comet on the morning of 6 August 1835, 3.3 months before perihelion passage. At that time the comet was 17 arc min in declination and 7 arc min in Right Ascension away from the predicted position, giving a predicted perihelion passage time 5 days too early.

So it seems that congratulations are in order. Yeomans' positional predictions have been proved to be extremely accurate, even though the comet is three times fainter than estimated. And the team who recovered the comet have pushed astronomical techniques to the limit and succeeded. All the scientists preparing space experiments for the 1986 perihelion passage of Halley's Comet can breathe a sigh of relief too. It is where it was expected to be, coming towards us at a speed of about 11 km s^{-1} ; they have just got 8.6 h less time to get ready. □

Confident carbon calibrations

from D.D. Harkness

SEVERAL proposals of fundamental importance to the confident interpretation of ^{14}C data were put forward at the 11th International Radiocarbon Conference*. Of major consequence was a plan to revise the constants and format used in the calculation and reporting of conventional ^{14}C years BP. In the event, the plan was overwhelmingly rejected in favour of the *status quo* — definition of the time scale by use of a half life of $5,570 \pm 30$ yr and the setting of AD 1950 as the zero age. Similarly, it was accepted that the expression of analytical uncertainty should remain as the individual laboratory's best estimate of one sigma statistical confidence in age measurement. In contrast, no agreement could be reached on the question of a standard procedure for isotopic normalization in the calculation of marine shell ages.

Routine accuracy in ^{14}C measurement was recently assessed in various laboratories on the basis of tree-ring dating (International Study Group *Nature* 298, 619; 1982) and a review of the results suggested the need for a more frequent exchange of samples between laboratories and the uninhibited publication of such results. Practicing laboratories also welcomed the opportunity for collective assessment of the new reference material (1980 oxalic acid) issued by the US

National Bureau of Standards. Following appraisal and discussion of its isotopic ratios, as measured relative to the established (1957 NBS oxalic acid) standard, the issue of the replacement standard was endorsed but it was noted that an accompanying statement on the isotopic enrichment factors proposed for routine dating work was desirable.

Causal mechanisms for variations in natural ^{14}C concentration received considerable attention and a consensus emerged that the established long-term and high-frequency (wiggle) variations in ^{14}C production rate primarily reflect changes in geomagnetic field intensity. Solar and climatic effects cannot be entirely precluded from the available tree-ring/ ^{14}C record but they are certainly small — no clear correlation is evident, for example, between the existing carbon isotope (tree-ring) record and historically recorded climate (M. Stuiver, University of Washington). Nevertheless, a measurable impact on the atmospheric ^{14}C inventory could be expected from the marked changes in the dynamics of the global CO_2 system during the glacial to postglacial transition (H. Oeschger, University of Berne). Beyond the range of dendrochronological comparison, parallel ^{14}C and ^{230}Th ages from cave deposits, which are concordant during the Holocene, indicate a higher ^{14}C content in the Peniglacial atmosphere — a major ^{14}C

*The 11th International Radiocarbon Conference was held at the University of Washington under the sponsorship of the National Science Foundation; the Weyerhaeuser Company and the Quaternary Research Centre, Department of Geological Sciences and Department of Physics, University of Washington. The conference proceedings will be published in *Radiocarbon* 25 (1983).

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fluctuation at about 29,000–31,000 BP is again attributable to variations in global magnetic field strength (J. Vogel, University of Pretoria).

Ongoing attempts to determine the precise secular pattern of past ^{14}C variations mainly make use of the opportunity provided by tree-ring data for 'absolute' calibration of ^{14}C ages. Significant progress has been made in compiling continuous ($\sim 8,000$ yr) chronologies from Irish and central European oak deposits with only a few gaps remaining to be bridged in each sequence (J. Pilcher, University of Belfast). Although numerous high-precision ^{14}C measurements with good agreement between laboratories are already completed from these oak deposits, an attitude of caution prevailed over early publication of the 'European oak calibration' as a more precise, but as yet partial, alternative to the re-evaluated Bristlecone pine record (Klein, J., Lerman, J.C., Damon, P.E. & Ralph, E.K. *Radiocarbon* 24, 103; 1982).

Several papers *en bloc* served to confirm the continuing role of $^{14}\text{C}/^{12}\text{C}$ variations, both natural and man induced, in studies of the contemporary environment. Appreciable coverage was given to the use of ^{14}C measurements, in conjunction with allied geochemical data, to establish the recharge, transport and carbon exchange characteristics of regional groundwater systems. The continued interest in ^{14}C as a quantitative tracer for mass transfer highlighted the importance of the deep ocean as the ultimate sink for fossil CO_2 ; however, the net uptake of this anthropogenic excess must lead eventually to the increased dissolution of sedimentary calcite (T. Peng, Oak Ridge National Laboratory). Akin to its value in identifying urban sources of atmospheric and freshwater pollution, the sensitivity of ^{14}C measurement can contribute to the direct evaluation of localized gaseous dispersion patterns from nuclear installations (R. Otlet *et al.*, AERE, Harwell).

Awareness of intrinsic contamination in sample materials selected for conventional dating studies was a recurrent theme. Organic-poor sediments, often preferred for their more lucid geomagnetic signature, are particularly susceptible to the 'ageing' influence of allochthonous carbon (J. King, University of Minnesota). Similarly, while ^{14}C analysis of freshwater carbonates can provide interesting geochemical insights, the inferred dates are, in general, suspect (R. Pardi, Queen's College, CUNY).

A comprehensive review of technical progress in the application of accelerator mass spectrometry to natural ^{14}C measurement came from some eleven research groups active in the field. In essence, while the great promise of a revolutionary breakthrough in ^{14}C dating method seems ever closer, it has yet to be

fulfilled. The routine preparation of suitable and isotopically homogeneous targets, together with the need to attain improved accelerator stability during and between sample runs, are outstanding constraints to the achievement of an analytical precision comparable with that established in radiometric counting. However, an incentive to continued development was the recent achievement of

about 5 per cent analytical precision (equivalent to ± 400 yr dating uncertainty) in $^{14}\text{C}/^{13}\text{C}$ measurement using milligramme-sized carbon samples (D. Donahue, University of Arizona). By present comparison the proven, if less instrumentally demanding, application of this analytical approach in natural ^{10}Be measurement represents an immediate and exciting prospect for Quaternary research.

From shilling prisms to digitized images

from L. T. Sharpe

SELDOM has the field of colour vision been more stimulating than it is today when new techniques allow us to explore what for centuries has been mere matter for speculation. Modern methods and historical contributions were jointly emphasized at a recent international meeting* on colour vision held in Cambridge — a fitting place to bring together colour scientists because the problems of colour have exercised many of the university's most distinguished physicists and physiologists, including Newton, Young, Maxwell and Lord Rayleigh. Indeed, for many at the conference, the beginnings of modern colour science were betokened by a holograph notebook (lent for display by the Fitzwilliam Museum) in which Newton recorded his purchase in 1667 of three prisms for three shillings.

The meeting covered an unusually wide range of topics from microspectrophotometry of visual receptor cells to the phenomenological constancy of coloured surfaces illuminated by spectrally very different sources.

Perhaps the most impressive advances have been made at the very beginning of the visual pathway, in studies of visual transduction in single primate photoreceptors by Nunn and Baylor of Stanford University that have just been reported in *Nature* (299, 726; 1982). Individual outer segments of rods from cynomolgus monkeys (*Macaca fascicularis*) were sucked into micropipettes of small ($2\mu\text{m}$) orifice diameter so that the reduction in membrane current that occurs when light stimulates the outer segment could be measured. Transverse stimulation of the outer segments by spectral lights over the range 400–720 nm produces a spectral sensitivity curve with a peak at 491 ± 3 nm. An interesting discrepancy is thus revealed — the result agrees well with

psychophysical measures of human scotopic sensitivity (if corrections are made for lens absorption and pigment self-screening) but not with microphotometric absorption measurements, where two independent groups find a peak value near to 500 nm. The difference recalls that seen between measurements of rhodopsin spectral sensitivity *in vivo* and in digitonin extract.

Only very preliminary results using the new technique are available for cones and for detailed information about their sensitivities we must rely mainly on microspectrophotometry. Recent technical refinements — transverse measurements that allow good optical isolation of the outer segment and yield reasonably high optical densities; digital computers that streamline data-gathering and analysis; and procedures for partial glutaraldehyde fixation of the retina that help maintain the structural integrity of the cone outer segments — have greatly increased the accuracy of microspectrophotometric data. Two separate groups, one in London (J. Bowmaker, H. Dartnall and J. Mollon), the other at Wood's Hole, Massachusetts (E. MacNichol Jr, J. Levine, R. Mansfield, L. Lipitz and B. Collins) are making precise measurements of the photopigments of individual rods and cones, using both fresh material and lightly fixed isolated retinæ. The two groups substantially agree about the shapes and locations of the λ_{max} of the major classes of pigment in the Old World monkey species (rhesus and cynomolgus). But residual differences exist concerning the properties of the short-wavelength-sensitive cones. The Wood's Hole group finds a longer λ_{max} for the rare short-wave cones of the cynomolgus monkey (429 ± 4 nm; $n=8$) than does the London group (415 nm; $n=2$). (Neither group finds any short-wave cones in the rhesus monkey.)

A new question is whether, within the normal human population, there are slight

*An international conference on colour vision was held at the University of Cambridge, 23–27 August 1982. The primary sponsor was the Scientific Affairs Division of NATO; additional support was provided by: The Royal Society, The Wellcome Trust and the US Army Research Institute for Behavioral and Social Science.

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variations between observers in the spectral positions of their photopigments. Factor analysis of Stiles and Burch's 10-degree colour-matching data suggests that there are (D. MacLeod and M. Webster). The standard deviation of peak absorption wavelength (for a given receptor class) among individuals is estimated between 1 and 1.5 nm. Human microspectrophotometric data suggest a somewhat larger variance. For instance, the London group showed that two 'normal' clinical patients (normal with respect to Rayleigh anomaloscope and Farnsworth-Munsell 100-hue results) had an 8 nm (553 and 561 nm) difference in the $\lambda_{\max}$ of their long-wavelength pigment sensitivities. A similar difference in long-wavelength sensitivity was found between the patients' field sensitivity ratios calculated at 555 and 650 nm. Even larger variations in the $\lambda_{\max}$ of the middle- and long-wave cones are found for the squirrel monkey, *Saimiri sciureus*. These too are well correlated with differences in behaviourally measured colour vision (G. Jacobs, University of California).

A new research tool is provided by digital computers that can control the display of digitized images on a colour television monitor. The way in which a given digital value is translated into a particular colour can be controlled and rapidly altered so that a stimulus can be moved in any direction in colour space. Despite some technical problems arising from instability, non-uniformity and flicker (W. Cowan, National Research Council, Ottawa and R. Rodieck, University of Washington), raster-based colour simulators can be impressively used (1) to simulate changes in the illumination of complex coloured scenes so that the visual computations underlying colour constancy may be analysed (J. McCann and K. Houston, Polaroid Corporation) and (2) to quantify the chromatic properties of neurones of the macaque lateral geniculate nucleus in terms of the secondary-stage processes or 'channels' of colour vision (A. Derrington, J. Krauskopf and P. Lennie, University of Sussex).

This last is very important because it is commonly assumed that visual information is transmitted centrally by two parallel and independent 'channels' — the achromatic and the chromatic. Although electrophysiology often appears to support this post-receptoral dichotomy, careful inspection of the properties of colour-coding retinal ganglion cells shows that their colour opponency is not a fixed characteristic, but rather varies with temporal (duration and frequency) and spatial (size and position) properties of the stimulus (E. Zrenner, Bad Nauheim). For instance, at low temporal frequencies (5 Hz), red-green colour opponent cells have two sensitivity maxima, one for excitation (say +R) and one for inhibition (say -G) with a broad neutral zone in between. At higher frequencies (30–33 Hz) colour



opponency is lost; the spectral sensitivity function becomes broadband and single-peaked, resembling that of luminance-detecting cells. The increase in sensitivity in the midspectral region seems to be based on a phase shift between the centre and surround responses which turns an antagonism into a synergism.

These results beg comparison with psychophysical experiments. Here too the simplistic view that it is possible to isolate 'channels' corresponding to two classes of neurone in the visual pathway is being challenged. The X-cells of the retina, whose small receptive fields predominate in the central fovea, are usually assumed both to code colour and to mediate resolution of acuity targets. When acuity is used as a criterion for equating the luminances of different colours, mixtures of such lights are photometrically additive. Additivity, in the conventional view, is a property of the luminance channel of psychophysics while the opponent chromatic channels are assumed to account for the nonadditivity of direct brightness matching and threshold measurements. How can the opponent red-green cells be nonadditive for threshold targets (detection of low spatial frequencies) and additive for acuity targets (detection of high spatial frequencies)?

All these complications suggest that colour and spatial vision may not be easily separable into discrete, processing channels. Indeed, even if separate channels exist for achromatic and chromatic vision, they may not be independent — luminance and chromaticity certainly interact in grating detection tasks. The interaction is not, however, symmetrical: colour masks luminance much more profoundly than luminance masks colour (K. De Valois and E. Switkes, University of California). The masking cannot be due purely to local (retinal) interactions because it is independent of relative test-mask phase. It must, therefore, occur at a level at which global chromatic and spatial frequency characteristics have been encoded.

Colour processing in the cortex is an area of hotly contested opinion. Two major controversies were discussed at the meeting. The first concerned whether particular parts of the primate prestriate cortex are specialized for colour analysis,

as has been proposed by S. Zeki (*Nature* 284, 412; 1980). Some researchers recording in anaesthetized and paralysed vervet monkeys find colour-coding cells dominating that part of area V4 lying on the lateral part of the anterior bank of the lunate sulcus (V. Andersen, C. Guld and O. Sjo, University of Copenhagen). Fewer are found in V2, almost none in V3. The colour-coding cells are often narrowly tuned for orientation (± 10 deg) and their spectral responses depend strongly on both the wavelengths of the adapting fields and the luminance of the stimulus. About half respond to white on a white background (usually a defining characteristic of luminance-coding cells). Other researchers, however, find few colour-coded cells in these areas when recording from alert, behaving rhesus monkeys (J. Krüger, B. Fischer and R. Boch, Freiburg I. Br.). Only about 10 per cent of the cells respond better to one or another colour. Rather they seem to have strong extraretinal inputs that are activated before and during visually guided eye movements. It may be, therefore, that in anaesthetized and paralysed animals extraretinal inputs cannot be activated and the excitability of the prelunate cortex depends only on cells with pure efferent sensory inputs originating from the retina. Thus for such 'unnatural' conditions the prelunate cells may merely appear to be colour biased.

The second controversy is whether colour cells in the primate striate cortex are confined to columns extending vertically from the cortical surface to the white matter (see also *News & Views* 300, 220; 1982). In the rhesus monkey, the columns are identified as slabs or stripes which are 100–250 μm wide and of indeterminate length (C. Michael, Yale University). Within a column, all neurones respond exclusively to monochromatic stimuli but differ in their receptive field organization, axis orientation, or eye preference. In the vervet monkey, however, there is no evidence for such colour columns (J. Krüger and M. Bach). Experiments in which $0.8 \times 0.64 \text{ mm}^2$ arrays of 30 parallel microelectrodes are used reveal non-colour-coding neighbouring sites in one cortical layer (say IVc) located directly above colour-coding sites in another layer (V). Such differences may be species dependent or may suggest that instead of colour columns encompassing the whole cortical thickness, independent columnar systems exist for the infra- and supra-granular layers. Before any more progress can be made on this issue, a consensus is needed on how to classify cells as colour coding. Some researchers classify cells as colour coding if they respond to coloured, but not white, stimuli; others if they respond differently to two colours irrespective of other stimulus parameters; and still others if they show a large difference in response strength to two colours for at least one out of several stimulus configurations. □

REVIEW ARTICLE

Origin, nature and world climate effect of Arctic Ocean ice-cover

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During the Cenozoic, an open water Arctic Ocean changed to the modern permanently ice-covered condition. Significant world climate modification accompanied this change but the precise role of the Arctic Ocean in major Pleistocene climate events is controversial. The present ice-cover averages 3 m thickness but there are theories that during the Pleistocene it was Antarctic-like, several thousand metres thick. The time of origin of the ice-cover is placed as young as 0.7 Myr ago and as old as the middle Miocene. Any theory concerned with origin, nature and climatic response of the Arctic ice-cover must consider the ocean's sediment record.

THE modern Arctic Ocean above 75° latitude has modest circulation with Pacific and Atlantic Oceans but is largely ice-covered during most of the year (Fig. 1). The detailed study necessary to understand Arctic Ocean history is not complete, in part, because of the logistical difficulties of working in an ice-covered ocean. Limited geophysical, oceanographic and biological studies have been accomplished even in the most inaccessible parts of the Ocean by use of the Arctic's ice as a platform from which to work. More than 500 short sediment cores that include the geological record of the central Arctic for parts of the Cretaceous to Holocene have been recovered from the ocean floor. Study of the sediment, its fauna and flora, provides data on sediment source, rate of sedimentation, water productivity, temperature and salinity and relationship of the Arctic water to the world ocean. In addition, aerial geophysical surveys have added to the Arctic Ocean data base¹. The limited data assembled from this work is important for understanding elements of geological and climatic development of the present Ocean as well as its role in the tectonic evolution of the Northern Hemisphere². Also, it is apparent that the Arctic Ocean has a significant role in the development of world climates during at least the past 75 Myr and may be an important factor in any future CO₂-induced climate change³.

During the Cenozoic, world climate changed from one with a low thermal gradient, only about 50% of that of the present, to the modern condition of strong gradients⁴. The oldest known Arctic Ocean sediment is late Cretaceous and it accumulated in an ice-free environment⁵. The absence of an Arctic ice-cover was an important factor contributing to the lower atmospheric

and oceanic thermal gradients that allowed temperate climates almost to reach the poles during the Cretaceous⁴. World-wide, these conditions changed sometime following the Cretaceous. The development of polar ice-covers probably dates from the Middle Cenozoic or late Neogene⁶ (Fig. 2). The Arctic Ocean sediment record is not complete for this period of time but glacial-marine sediment was accumulating at least by the late Miocene in the central Arctic⁷. Together with Antarctic ice development, an Arctic ice-cover produced a profoundly steeper thermal gradient than had existed during the Mesozoic. This was a factor in the initiation of Pleistocene glaciation and modern climate.

The evidence for the Arctic's role in the late Cretaceous to middle Cenozoic climate change is fairly well understood. The Arctic sediment record is one of biogenic silica, diatoms and silicoflagellates, probably representing relatively warm upwelling conditions during the late Cretaceous⁵. This was followed by a change to glacial-marine sedimentation for at least the late Miocene to Holocene⁷. The sediment record for the entire late Cenozoic Arctic Ocean indicates that more or less continuous glacial ice-rafting has been the principal sedimentation process. There were times when ice-rafting was very intense, alternating with time of less clastic sedimentation⁷. During these intervals of limited ice-rafting, increased biological productivity is suggested by increase in foraminifera, ostracodes and calcareous dinoflagellates. These data must be considered part of any theory that attempts to explain the nature of the Arctic ice-cover although it has not been used by those who have proposed fluctuating climatic regimes or giant ice-caps for the late Cenozoic central Arctic Ocean. Also while the data on sediment types are unequivocal, the timing of the development of the first Arctic ice-cover as well as the precise sequence of events leading to modern conditions are issues not fully resolved. The 'fine tuning' for resolution of time of initiation of the Arctic ice-cover may not be accomplished until a very complete central Arctic Ocean sediment core is recovered. Meanwhile based on short sediment core study, the discussion of the palaeoclimatologic changes that accompanied the development of the modern Arctic Ocean has produced diverse ideas.

Arctic role in the world climate

The importance of the Arctic Ocean for world climate was recognized by the 1956 Ewing-Donn theory⁸. In this early attempt at an unified explanation for Pleistocene glacial cycles, the Arctic Ocean had a central role. According to the theory, an open, ice-free ocean produced moisture to support the expansion of glaciers. Melting and retreat of glaciers in lower latitudes occurred when an Arctic Ocean ice-cover formed, sealing off the source of moisture and thus affecting glacier

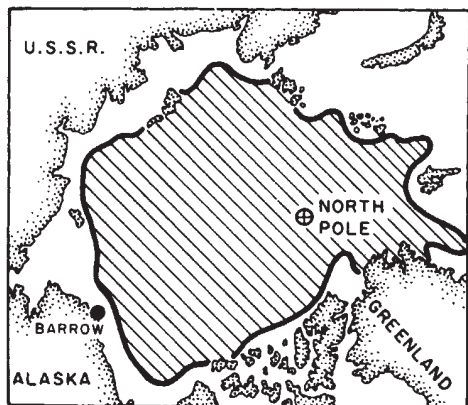


Fig. 1 Approximate minimum extent of permanent Arctic Ocean ice-cover, early autumn.



Fig. 2 The modern Arctic Ocean ice-cover. Aerial view showing dark, fine grained sediment laden pack-ice. Irregular pattern of sediment-rich ice and cleaner pack-ice due to fracturing of pack-ice during movement.

growth. In this ingenious theory, glacial and interglacial intervals of the Pleistocene were thought to be highly dependent on the condition of the Arctic Ocean surface.

Confirmation of the validity of the Ewing–Donn theory could be tested in various ways. In the years following its publication, palaeontologists, marine geologists and others gathered diverse data that questioned many of the theory's basic tenants. Using pollen data from the area around the Arctic basin, Colinvaux^{9,10} summarized objections to the theory based on ecological constraints of demonstrated floral occurrences. Next, sediments and sedimentation rates in Arctic cores were found to be inconsistent with the theory^{11–13}. Foraminifera abundance patterns for the central Arctic during the past 1.5 Myr showed no time of ice-free conditions that could be correlated with glacial or interglacial stages¹⁴. This raised additional objections to the theory and it was concluded that the Arctic must have remained frozen while lower latitude Pleistocene glacial advances and retreats were in progress¹⁴. Alternating thickening or thinning of the ice-cover may have occurred during the Pleistocene but the sediment, floral and faunal record seemed to confirm that there was no evidence for an open Arctic Ocean that could be correlated with Pleistocene glacier growth. Thus, the Ewing–Donn theory was untenable.

If the Arctic ice-cover was not cyclic in appearance and disappearance during the Pleistocene, (and it was ice-free in the earliest Cenozoic) the obvious question was the time when the Arctic Ocean first become ice-covered. Also, what were the changes that accompanied the great freeze and what was the condition of the Arctic Ocean throughout the Cenozoic? Although these questions could be answered by study of a continual sediment record for the Arctic Ocean, no DSDP Leg has been planned for the central Arctic and the ice problems are so profound that no similar drilling program is contemplated. However, extensive coring from floating ice-island T-3, produced several short sediment cores and their study produced good data. Most of the cores recovered during the T-3 years (1955–74), some 580 successful casts, were taken as part of heat flow studies by the US Geological Survey (A. Lachenbruch and V. Marshall). After conductivity measurements, sediment and faunal studies of the cores were undertaken at the University of Wisconsin-Madison. In addition several cores were taken by a group at Lamont-Doherty Laboratory (under K. Hunkins) and became the basis for work at Lamont as well as later work at Washington State University. The Arctic sediment cores yielded a wealth of data that were subsequently used in faunal analysis^{12,14–24}, sediment studies^{7,25–28}, and became the basis for diverse palaeoenvironmental interpretations^{14,29–34}. These environmental interpretations have assumed new importance because of theoretical considerations related to a possible future CO₂ induced world climate change³. If a climate change occurs,

one of the first effects may be the disappearance of at least the summer Arctic ice-cover³. In order to understand the environmental impact of such an event, climatologists have considered the possibility that there was a time in the recent geological past (past 3 Myr) when the Arctic had no ice-cover. If such a time occurred, it could provide an accurate analogue for understanding the change that might occur in the future³.

There is no uniformity of thought concerning conditions of the Arctic ice-cover during the past 3 Myr. From the various data based interpretations as well as theoretical considerations, three different theories have been proposed to explain the evolution of the modern Arctic Ocean: the (1) fluctuating regime and (2) steady state theories are based on different interpretations of central Arctic sediment cores; the (3) ice-cap theory is based on data more theoretical, and from outside of the Arctic Ocean.

Formation of the modern Arctic Ocean

Each of the three theories, presumably includes an evolution of the present ice-cover, from similar beginnings but through quite different stages of development.

(1) The 'fluctuating regime' theory^{35–37} proposes that the late Miocene to Holocene interval was represented by three different palaeoclimatologic regimes, only the most recent of which (I), occurring from approximately 0.7 Myr to Recent, was characterized by a perennial ice-cover. Of the three theories, this is the most elaborate and involved. This theory suggests that during palaeoclimatologic regime III (interpreted to be roughly from 4 or 5 Myr ago to 2.7 Myr ago), the Arctic Ocean was cold but not ice-covered. The ocean had productive waters, interpreted by the theory's proponents to be the highest productivity of any time during the past 4.5 Myr. The productivity of the surface waters affected deeper waters, as well, because of good vertical mixing. The next younger or palaeoclimatologic regime II, is interpreted to be characterized by stratified saline and fresh water with little vertical mixing, resulting in both little surface productivity as well as no bottom productivity. Coccoliths were reported to occur during this interval³⁶ and ice-free conditions are suggested, at least for part of the year. This regime extended from 2.7 to 0.7 Myr ago. Finally, the most recent climate regime, I, is interpreted to have been initiated at about the same time as the Brunhes–Matuyama boundary (0.7 Myr), and at this time permanent ice, similar to that of the present, formed. This time interval is interpreted to be characterized by sharp and frequent fluctuations in foraminifera abundance, and sparse coccoliths. Sparsity of coccoliths is suggested to be due to intense bioturbation.



Fig. 3 Layers of fine silt and clay exposed in pack-ice that has been thrust in a pressure ridge. Fine grained sediment is incorporated into newly forming ice during autumn storms in shallow shelf areas and then drifts seaward with surface currents. Pack-ice is a major source of fine grained sedimentation in the central Arctic Ocean.



Fig. 4 Iceberg, moving with pack-ice of Arctic ice-cover, and showing glacial derived coarse grained sediment layers. Icebergs are the only source of coarse grained sedimentation in the central Arctic Ocean.

While, elaborate in design, many of the conditions interpreted to be necessary for this theory have a questionable basis. This may be due to the fact that fewer than a dozen dried-out and heavily sampled sediment cores from the limited Lamont collections were available for study. For example, strong vertical circulation and high productivity, interpreted for climate regime III, is based on abundance of Fe-Mn micronodules that are said to have foraminifera as nuclei. These micronodules, reported to characterize only regime III³⁵, actually occur in moderate abundance throughout the cores (sediment of regimes I and II, as well) and most do not have foraminifera as nuclei^{7,19}. Regime II, said to have resulted from strongly stratified waters with little or not organic productivity, actually spans the time of the beginning of abundant foraminifera and, in general, the Arctic Ocean was more productive during this time than during time of regime III. O'Neill¹⁹ studied 11 fresh sediment cores from along the flanks of the Alpha Cordillera and Canada Basin, some at 3–4 cm intervals. He recognized 75 benthic foraminifera species including 15 textulariid and miliolid species and 34 rotalid species, that range through the interval of regime II, said to be a time of nonproductive waters^{35–37}.

After comparing the fluctuating regime theory with the data of his cores, O'Neill concluded that the "high productivity model suggested by Herman and Hopkin³⁵, (for regime III) does not appear to be supported by the available data".

Inferences of red clay occurrence and bioturbation to depths of 30 cm during any of the regimes also cannot be verified^{7,19}. Rather, glacial-marine sediment with erratics characterizes all sediment (from at least 5 Myr ago) and bioturbation cannot be interpreted to have affected more than 10 cm of sediment. Inconsistencies in the interpretation of coccolith data are also worrying. In July and August of 1980, Margolis and Herman³⁷ and Herman and Hopkins³⁵ reported that coccoliths occur in regime III sediment, but in October³⁶ no coccoliths nor discoasters were reported for regime III. In the final report³⁶, it turned out that 5 of the 6 samples said to have had nannofossils actually occurred in regime I sediment; the sixth sample (with two specimens) was from regime II. This was used as part of the evidence for ice-free condition. Ice-rafting of an occasional coccolith from marginal Arctic waters to the central Arctic may be a better explanation for all of the occurrences.

The fluctuating regime theory is difficult to verify because of inconsistencies in the reports as well as apparent misinterpretation of the sediment cores that form the basis for the theory. (2) The 'steady state' theory^{7,13,33,34} is also sediment based and argues that the same kind of glacial-marine sediment that accumulates today under an average of 3 m ice, has been accumulating for at least 5 Myr. Although there have been some changes in rate of sedimentation and in percentages of coarse material deposited, the changes have been of the same magnitude during most of the past 5 Myr and probably represent

sedimentation in more or less similar conditions. The sediment record for several hundred cores has been organized into a lithostratigraphical sequence of 13 stratigraphical units⁷. The stratigraphical units consist of silty lutites reflecting lower sedimentation rates and corresponding to decreased ice-rafting, alternating with arenaceous lutites representing higher sedimentation rates and corresponding with increased time of ice-rafting. Clays and silts are transported both by pack-ice (frozen seawater) (Figs 2, 3) and glacial-ice (icebergs) but coarser sediment is only transported by icebergs³⁸ (Fig. 4). The lithostratigraphical sequence includes units that are fine-grained (probably principally rafted by pack-ice) and coarse-grained (rafted by glacial-ice). Because these units alternate throughout the 5 Myr for which the central Arctic record is more or less complete, both pack-ice and glacial-ice are considered to have been present since at least the Miocene. Older Cretaceous–Palaeocene material was recovered in two cores and their study was the basis for the interpretation that open water conditions existed during the late Mesozoic to early Cenozoic⁵. No sediment record has been recovered for the Eocene to late Miocene interval.

The steady state theory interprets the sediment record of the Arctic Ocean for the past 5 Myr to show little variation on a single theme, ice-rafting. Foraminifera and calcareous dinoflagellates show positive correlation during part of the Pleistocene (Fig. 5). Foraminifera abundance in the central Arctic Ocean and a percentage of coarse, glacial-ice-rafted material show inverse correlations throughout the interval when significant foraminifera have been present. Some effect of dilution by clastic influx is possible. Calcareous dinoflagellate occurrence also shows negative correlation with increased glacial ice-rafting³⁹ (Fig. 6). This is interpreted to indicate that during rapid ice decay and calving (inter-glacial intervals), surface waters may have been too low salinity to support foraminifera and dinoflagellates but that these organisms were relatively productive during stable glacial-growth (glacial intervals). The surface of the central Arctic Ocean could have been perennially ice-covered during the entire interval.

A fine tuning of this theory has resulted from the study of calcareous dinoflagellate fossils that show alternating times of abundance and rareness during the interval 2.0–0.7 Myr ago. Evidently, during this 1.3-Myr period, favourable conditions to encourage production of calcispheres occurred. Conditions before 2.0 Myr and after 0.7 Myr have been more like the present³⁹.

(3) The 'ice-cap' theory argues that Pleistocene sea level changes and oxygen isotope records from benthic foraminifera of the lower latitude oceans could be more easily explained if the Arctic Ocean had an Antarctic size ice-cap 1,000 m thick, during all or most of the Pleistocene^{40–44}. This theory has evolved from ideas based on a West Antarctic analogy and explanations for displaced marine fossils in Alaska⁴⁰ and theoretical considerations suggesting that during the past 160,000 yr,

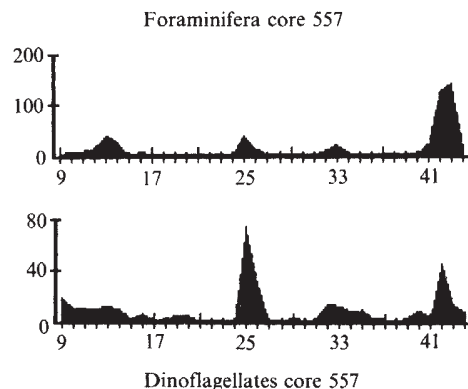


Fig. 5 Abundance of foraminifera and positive correlation with abundance of dinoflagellates for the interval 2.0–0.7 Myr ago in the central Arctic in core F1-557. Notice different vertical scales.

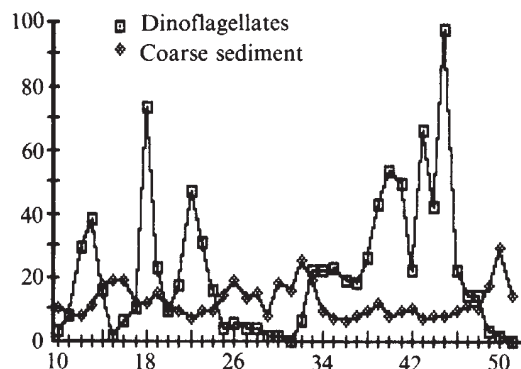


Fig. 6 Plot of abundance of dinoflagellates and percentage of coarse (glacially derived) sediment for core F1-301, central Arctic Ocean. General negative correlations seen in all cores studied, that is, time of occurrence of greatest percentage of coarse sediment (sample 50) correlates with time of lowest number of dinoflagellates and vice-versa (sample 45, 22 and so on).

the world benthic foraminifera ^{18}O record requires a larger ice volume than can be interpreted from sea level data^{43,44}. The ice needed to explain the apparent discrepancy between sea level calculation and ^{18}O records of ice volume is interpreted to have been in the Arctic Ocean. A 50-m sea level difference, that would account for at least some of the discrepancy, would require a floating ice cap 1,300 m or more thick "close to the mean depth" of the Arctic Ocean⁴⁴. Unfortunately, this theory ignores data from the Arctic Ocean. As pointed out earlier, during most of the 160,000 yr that a 1,000-m ice sheet is theorized to have been present, sedimentation similar to that of the modern Arctic ocean was in progress and various types of organisms lived, and at times, even flourished. It is difficult to understand phytoplankton and foraminifera thriving under >1,000 m of ice, as the ice cap theory demands. Clearly the evidence for relatively high organic productivity based on hundreds of short sediment cores that include the critical time interval, cannot be ignored^{14,19}. Similarly, the mechanism that would permit accumulation of ice-rafted sediment beneath a >1,000 m ice-cap whose thickness is close to the mean depth of the ocean, is unknown.

This theory must accommodate the extensive data from Central Arctic Ocean sediment cores as well as theoretical considerations before it can be used to explain Pleistocene events.

Future CO_2 -induced climate change and Arctic Ocean ice-cover

The three theories are concerned with the modern Arctic Ocean ice-cover and its geological history. Climatologists worried about the increasing atmospheric CO_2 are concerned about the melting of the Arctic ice cover⁴⁵⁻⁴⁷ and future climatic effects. The early disappearance of the perennial Arctic ice-cover during a CO_2 -induced climate change would produce a unipolar cryosphere, affecting sea surface temperature and producing a shift of climatic belts by several degrees latitude³. The climatic impact of an ice-free Arctic Ocean has received much consideration^{48,49} although a sea level rise perhaps mostly related to Antarctic melting, may be in progress⁵⁰.

Flohn³ has speculated that the best understanding of future effects on the climate could be obtained from a geological analogue; a time of Arctic Ocean ice-free conditions with continental positions and Earth climate of the same general magnitude as that of the present. That such a time did actually exist is disputed and it is argued that modelling of an ice-free Arctic may be the superior method of predicting climatic effects⁵¹.

There is no widespread agreement that a realistic model of an ice-free Arctic Ocean has actually been produced, although there have been several interesting attempts⁵²⁻⁵⁸. Several of the models suggest that the ice could melt in the summer but even under considerable atmosphere CO_2 addition, winter ice would form, at least as long as the Earth's current orbital status persists. The climate change produced by an absence of summer ice and a reduced winter ice cover are matters for atmosphere modellers. No geological analogue with continents and climate patterns such as those of the present, has probably emerged for 5 Myr.

Conclusions

The role of the Arctic Ocean in Earth climate patterns has been significant, at least for the past 100 Myr. An open Arctic Ocean with extensive upwelling and free exchange with more southerly waters characterized the late Cretaceous-early Cenozoic. This is confirmed from central Arctic sediment^{5,20,21} as well as from vertebrate and plant study in Arctic and sub-Arctic areas⁵⁹. Formation of the Antarctic ice sheets along with development of glacial conditions in the Arctic were important factors for the general cooling of Earth during the Middle Cenozoic. Glacial-marine sediment was forming in the central Arctic at least 5 Myr ago. The first freezing of the Arctic surface waters may have been earlier than this but there is no direct sediment evidence to support this idea. This age probably is a minimum, as ice-rafted debris may have been accumulating in Svalbard as early as the Eocene⁶⁰.

From at least 5 Myr ago to the present, glacial-marine sediment, transported by ice-rafting of icebergs and pack-ice, has accumulated in the central Arctic Ocean. Generally, times of extensive ice calving are correlated with accumulation of coarser sediment and abundant erratics. These intervals of relatively rapid sedimentation (for the Arctic) alternated with times of accumulation of finer grained, predominantly ice-pack transported sediment and greater organic productivity. From ~2 to 0.7 Myr, calcareous dinoflagellates left a record of alternating abundances and rareness. Probably, during this time surface conditions permitted greater productivity than had been possible before 2 or since 0.7 Myr.

There is no evidence from the Arctic Ocean to support the idea of giant Ice Caps, 1,000 m thick, during any part of the Pleistocene. Evidence from modelling for future CO_2 induced climate changes predicts a reduced winter ice-cover and an absent summer ice-cover for the Arctic Ocean. If this occurs, significant climate changes are indicated. Unfortunately, no geological analogue is available. Rather, the only evidence from the central Arctic, suggests that conditions like those of the present, punctuated by times of greater iceberg activity, characterized the Arctic Ocean during most of the late Cenozoic.

Photographs are courtesy of Arnold Hanson, University of Washington.

- Vogt, P. R., Taylor, P. T., Kovacs, L. & Johnson, G. L. *J. geophys. Res.* **84**, 1071-1089 (1979).
- Churkin, M. & Trexler, J. H. *The Ocean Basins and Margins Vol. 5 The Arctic Ocean*, 1-20 (Plenum, New York, 1982).
- Flohn, H. *CO₂ Review* 145-179 (Cambridge, New York, 1982).
- Frakes, L. A. *Climates throughout Geologic Time* (Elsevier, Amsterdam 1979).
- Kitchell, J. A. & Clark, D. L. *Paleogeogr. Paleoclimatol. Paleocol.* (in the press).
- Berggrán, W. A. & van Couvering, J. A. *The Late Neogene* (Elsevier, Amsterdam, 1974).
- Clark, D. L., Whitman, R. R., Morgan, K. A. & Mackey, S. D. *Geol. Soc. Am. Spec. Pap.* **181**, 57p (1980).
- Ewing, M. & Donn, W. L. *Science* **123**, 1061-1066 (1956).
- Collinvaux, P. A. *Science* **145**, 707-708 (1964).
- Collinvaux, P. A. in *The Bering Land Bridge*, 207-231 (Stanford, California, 1967).
- Ku, T. L. & Broecker, W. S. *Progr. Oceanogr.* **4**, 95-104 (1967).
- Steuerwald, B. A., Clark, D. L. & Andrew, J. *Earth planet. Sci. Lett.* **5**, 79-85 (1968).
- Clark, D. L. *Arctic* **22**, 233-245 (1969).

- Clark, D. L. *Bull. geol. Soc. Am.* **82**, 3313-3323 (1971).
- Lagoe, M. B. *Bull. geol. Soc. Am.* **87**, 1678-1683 (1976).
- Joy, J. A. & Clark, D. L. *Micropaleontology* **23**, 129-154 (1977).
- Lagoe, M. B. *J. Foraminifera Res.* **7**, 106-129 (1977).
- Gamber, J. H. & Clark, D. L. *Micropaleontology* **24**, 422-431 (1978).
- O'Neill, B. J. *J. Paleont.* **55**, 1141-1170 (1981).
- Bukry, D. *Geo-Marine Lett.* **1**, 57-63 (1981).
- Ling, H. Y., McPherson, L. M. & Clark, D. L. *Science* **180**, 1360-1361 (1973).
- Steuerwald, B. & Clark, D. L. *J. Paleont.* **46**, 573-580 (1972).
- Ericson, D. B., Ewing, M. & Wollin, G. *Science* **144**, 1183-1192 (1964).
- Herman, Y. *Nature* **201**, 386-387 (1964).
- Hunkins, K. & Kutschale, H. *Prog. Oceanogr.* **4**, 89-94 (1967).
- Clark, D. L. *Bull. geol. Soc. Am.* **81**, 3129-3134 (1970).
- Mullen, R. E., Darby, D. A. & Clark, D. L. *Bull. geol. Soc. Am.* **83**, 205-212 (1972).
- Campbell, J. S. & Clark, D. L. *J. sedim. Petrol.* **47**, 657-670 (1977).
- Herman, Y. *Paleogeogr. Paleoclimatol. Paleocol.* **6**, 251-276 (1969).

30. Herman, Y. *Science* **169**, 474–477 (1970).
31. Hunkins, K., Be, A. W. H., Opdyke, N. D. & Mathieu, G. *The Late Glacial Ages*, 215–237 (Yale, Connecticut, 1971).
32. Herman, Y. in *Marine Geology and Oceanography of the Arctic Seas*, 283–348 (Springer, New York, 1974).
33. Clark, D. L. in *Polar Oceans*, 603–615 (Arctic Institute of North America, Montreal, 1977).
34. Clark, D. L. *Biol. Geol.* **2**, 55–76 (1977).
35. Herman, Y. & Hopkins, D. M. *Science* **209**, 557–562 (1980).
36. Worsley, T. R. & Herman, Y. *Science* **210**, 323–325 (1980).
37. Margolis, S. V. & Herman, Y. *Nature* **286**, 145–149 (1980).
38. Clark, D. L. *Bull. Am. Ass. petrol. Geol.* **65**, 911 (1981).
39. Gilbert, M. W. & Clark, D. L. *Mar. Micropaleont.* (in the press).
40. Mercer, J. H. *Paleogeogr. Paleoclimatol. Paleocool.* **8**, 19–27 (1970).
41. Broecker, W. S. *Science* **188**, 1116–1118 (1975).
42. Hughes, T., Denton, G. H. & Grosswald, M. G. *Nature* **266**, 596–602 (1977).
43. Williams, D. F., Moore, W. S. & Fillon, R. H. *Earth planet. Sci. Lett.* **56**, 157–166 (1981).
44. Keigwin, L. D. *Nature* **296**, 808–809 (1982).
45. Budyko, M. I. *Izv. Akad. Nauk. SSR Ser. Geogr.* **6**, 3–10 (1962).
46. Budyko, M. I. *Tellus* **21**, 611–619 (1969).
47. Budyko, M. I. *Izmeneniia Klimata* (Leningrad, 1977, transl. Zolina, R.) (American Geophysical Union).
48. Kukla, G. & Gavin, J. *Science* **214**, 497–503 (1981).
49. Hansen, J., Johnson, D., Lacis, A., Lebedeff, S., Lee, P., Rind, D. & Russell, G. *Science* **213**, 957–966 (1981).
50. Gornitz, V., Lebedeff, S. & Hansen, J. *Science* **215**, 1611–1614 (1982).
51. Clark, D. L. *CO₂ Review*, 181–184 (Cambridge, New York, 1982).
52. Rakupova, L. R. *Proc. Symp. on the Arctic Heat Budget and Atmospheric Circulation*, 411–441 (Memo. RM-5233-NSF, Rand Corp., California, 1966).
53. Donn, W. L. & Shaw, D. M. *J. geophys. Res.* **71**, 1087–1095 (1966).
54. Manabe, S. & Stouffer, R. J. *Nature* **282**, 491–493 (1979).
55. Manabe, S. & Stouffer, R. J. *J. geophys. Res.* **85**, 5529–5554 (1980).
56. Parkinson, C. L. & Kellogg, W. W. *Climate Change* **2**, 149–162 (1979).
57. Manabe, S. & Wetherald, R. T. *J. atmos. Sci.* **32**, 3–15 (1975).
58. Thompson, S. L. & Schneider, S. H. *Nature* **290**, 9–10 (1981).
59. West, R. M., Dawson, M. R. & Hutchison, J. H. *Biol. Geol.* **2**, 77–93 (1977).
60. Dalland, A. *Norsk Polar. Arbok* 151–165 (1976).

ARTICLES

A voltage-gated ion channel model inferred from the crystal structure of alamethicin at 1.5-Å resolution

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The crystal structure of alamethicin in nonaqueous solvent has been determined, and refined at 1.5-Å resolution. The molecular conformation of the three crystallographically independent molecules is largely α -helical with a bend in the helix axis at an internal proline residue. The helix structure is highly amphipathic as most of the solvent-accessible polar atoms lie on a narrow strip of surface parallel to the helix axis. Molecular models for the voltage-gated ion channel, with n-fold symmetry and based on the molecular conformations observed in the crystal, are characterized by strong surface complementarity, a hydrophilic interior and a hydrophobic exterior. The channel structures are stabilized by a hydrated annulus of hydrogen-bonded glutamine residues which produce the greatest restriction in the channel diameter.

THE linear peptide antibiotic alamethicin spontaneously inserts into black lipid membranes producing voltage-gated ion channels¹. While alamethicin isolated from *Trichoderma viride* is a mixture of several related chemical species^{2,3}, the sequences of the major (and minor) components have been identified as^{4,5}:

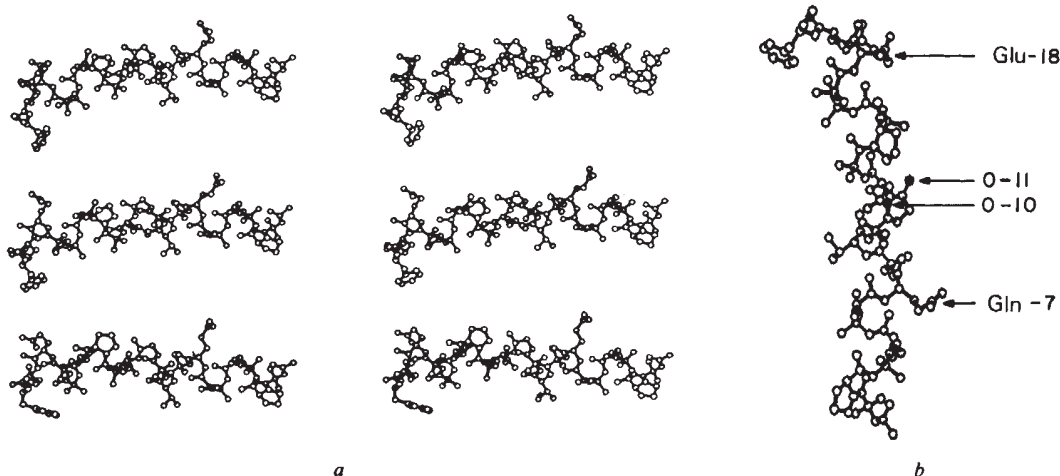


although cyclic^{2,6} and branched⁷ structures have been proposed. The N-terminal residue of alamethicin is acetylated (Ac⁺). The peptide contains eight or nine α -aminoisobutyric acid (Aib) residues and has L-phenylalaninol (Phl) as its C-terminal

residue. The conductance properties of alamethicin in black lipid membranes have been extensively investigated and recently reviewed⁸. The macroscopic conductance is strongly voltage-dependent, increasing e -fold for every 4–6 mV in applied voltage, and also exhibits a strong alamethicin concentration and ionic strength dependence^{1,9–12}. The high power dependence of the conductance on alamethicin concentration suggests that the alamethicin ion channel consists of 6–11 or more molecules. An ionic strength-dependent partitioning of alamethicin into a lipid phase appears to explain the observed dependence of conductance on salt concentration¹⁰.

At low alamethicin concentrations, conductance fluctuations are observed which have been attributed to single-channel gating events^{9,13,14}. Single-channel current bursts fluctuate

Fig. 1 *a*, The three alamethicin molecules present in the asymmetric unit are positioned in a similar orientation to the N-terminal segment. The N-terminus is to the right in each stereo diagram. The hydrophilic surface of each molecule projects towards the top of the figure. *b*, The solvent-accessible polar groups (see text) are labelled for molecule I. The hydrophilic surface of the molecule projects to the right.



among several discrete conductance levels which are not integral multiples of each other. The most probable conductance substate and the lifetime of the current burst are only moderately voltage-dependent. The strong voltage dependence of the macroscopic conductance has been attributed to a change in the number of gated channels rather than a significant change in the properties of a single channel. The alamethicin channel is only weakly cation-selective^{13,15-17}. The higher conductance substates are less sensitive to cation radius¹⁶, indicating that the increase in current is due to a change in the cross-section of the channel, rather than an increase in the number of parallel open channels of similar area. Kinetic measurements of alamethicin-induced current are well modelled by a single exponential time constant, which is only moderately dependent on the alamethicin concentration in phosphatidylcholine membranes¹¹ and independent of peptide concentration in glycerol monooleate membranes¹². These observations suggest that alamethicin exists predominantly in a preformed oligomeric state before a voltage is applied to the membrane.

The voltage-gating event must represent the movement of a charged group in the electric field or the orientation of a molecular dipole. The latter mechanism has been accepted in most models of alamethicin channel voltage-gating. The 'barrel stave' model of alamethicin action^{14,18} suggests that peptide monomers at the surface of the alkyl chain region of the bilayer, are oriented by the electric field via coupling to the molecular dipole, and aggregate to form an oligomer once aligned with the electric field. The conductance substates are explained by a change in the channel cross-section due to a fluctuation in the number of monomer units in the oligomer. Hall¹⁹ has proposed a two-dimensional micelle of alamethicin at the surface of the membrane which is oriented in the electric field via the molecular dipole, forming an oligomeric structure having a constant number of monomers, which fluctuates in an undefined way to generate the conductance substates.

The conformation of alamethicin is partially α -helical in organic solvents, as determined by circular dichroism^{20,21}. Crystal structures of small peptide fragments of alamethicin and poly(α -aminoisobutyric acid) are in a 3_{10} helical conformation^{22,23}, although a larger Aib-containing peptide adopts an α -helical conformation in crystals²⁴.

We performed a crystallographic analysis of alamethicin to define the detailed molecular conformation and packing involved in the ion channel, and to differentiate among the proposed chemical structures^{2,4-7}. This structure analysis has defined the helix type (α or 3_{10}) and the deviations from those ideal geometries, and illustrated the packing of Aib-containing helices. Knowledge of the molecular structure in the crystal has allowed model-building studies to define the proposed channel structure.

Structure determination and refinement

Alamethicin was provided by the Upjohn Company and was used without further purification²⁵. Crystals suitable for diffraction were prepared by 10-fold dilution with acetonitrile of a solution of alamethicin in methanol (100 mg ml⁻¹). Integrated X-ray diffraction intensities were collected to 1.5 Å resolution

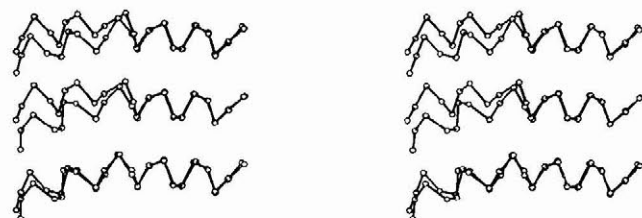


Fig. 2 The alamethicin molecules have been oriented as in Fig. 1, but are represented here as a stereo diagram of the α -carbon traces. The molecules are oriented such that the distance between α -carbon positions over the first 10 residues is minimized. The acetyl methyl group has been included as the first α -carbon. The molecular pairing from top to bottom is I-III, II-III, I-II.

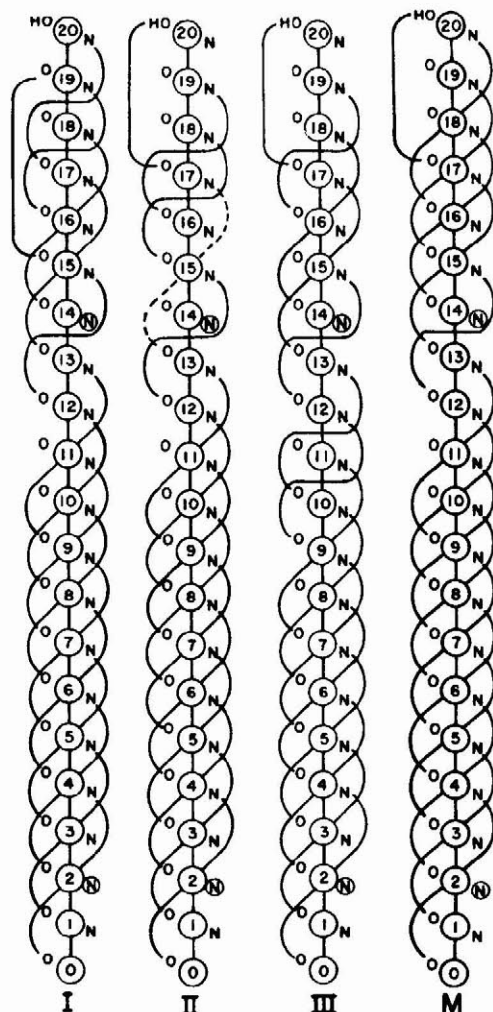


Fig. 3 The intramolecular hydrogen-bonding pattern for each of the three independent alamethicin molecules (I, II, III) is indicated together with the connections used in the channel model building (M) as described in the text. Residue 0 represents the acetyl group; circled N symbols, proline residues. The broken lines indicate an unusually long interaction. Hydrogen bonds of the 5 to 1 type (α -helix) cross the vertical diagonally while 4 to 1 hydrogen bonds (3_{10} helix) are represented by lines crossing the vertical axis horizontally.

by diffractometry using a modified Wyckoff scan²⁶. An isomorphous heavy-atom derivative was prepared by diffusion of dimethyl mercury into the crystal from a mother liquor solution. Heavy-atom parameters and protein phase angles were refined²⁷ at 3.0 Å resolution. An atomic interpretation of the electron density map was constructed and refined using the distance-restrained least-squares procedure of Konnert and Hendrickson²⁸. Calculated phases from the refined model were used to extend the resolution from 3.0 Å to 1.5 Å.

Molecular models of the proposed channel structures were constructed with Corey-Pauling-Koltun (CPK) units and by a molecular graphics program using an Evans and Sutherland PSII display system. Space-filling drawings were generated on a colour raster graphics unit (Advanced Electronics Design, Inc.) using the program of Connolly²⁹.

Crystals of alamethicin are of space group P2₁, $a = 33.33$, $b = 29.62$, $c = 23.20$ Å, $\beta = 120.4^\circ$ and contain three molecules per asymmetric unit. The solvent content of the crystals is 30%. The present analysis was carried out at 1.5 Å resolution although diffraction maxima have been observed to 1.0 Å. The distance-restrained least-square refinement converged with a crystallographic residual of 15.5% and a r.m.s. deviation from ideal bond lengths of 0.04 Å. Details of the crystal structure determination and refinement will be presented elsewhere.

Molecular structure and crystal packing

The crystal structure of alamethicin is consistent with the linear backbone structure^{4,5}, and excludes the cyclic^{2,6} and branched⁷ structures from further consideration. The side chain density of the final ($2F_o - F_c$), model-phased, electron density map, calculated from the complete molecular model, reveals no deviation from the chemical sequence proposed for the major component of alamethicin⁵. It is unlikely that side chain heterogeneity due to minor components, if they were in fact present in the crystals, would be observed in the electron density map described above.

In general, all three alamethicin molecules in the asymmetric unit exist in an α -helical conformation over their entire lengths. There are, however, small deviations from a continuous α -helical conformation in the form of short (one to two residues) 3_{10} helical regions in the C-terminal domain. Figure 1 shows a stereo diagram of the three independent molecules. Each helix axis is bent slightly and to a different extent near the Pro-14 residues. The angular variability of the bend and the variations in helix parameters seem to be due to the differences in intermolecular polar interactions among the three independent alamethicin molecules. Figure 2 illustrates the difference in bend angles among the three molecules (I, II, III), and Fig. 3 gives the intramolecular hydrogen-bonding pattern for each molecule.

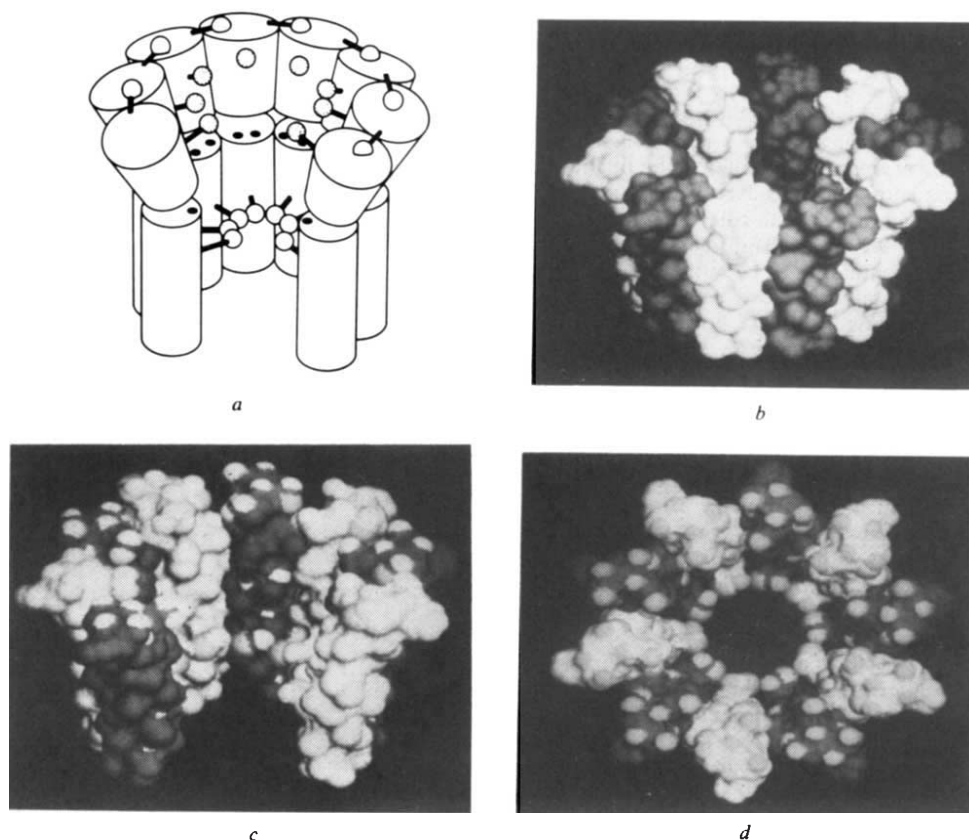
The accommodation of the Pro 14 residue into the α -helical backbone is accomplished by the introduction of a $4 \rightarrow 1$ hydrogen bond (N-15 to O-12) which shifts the proline imino ring from a position directly above the carbonyl of Aib 10 to one between the carbonyls of Aib 10 and Gly 11. The Pro 14 imino ring is further accommodated by the bend in the helix axis away from the Pro 14 ring direction. In Fig. 2 the molecules have been superposed such that the overlap of like α -carbons for the first 10 residues is maximized between each pair of

molecules. Molecules I and II display a similar bend angle, deviating significantly only at the C-terminal residues where the hydrogen-bonding pattern differs. Molecule III displays a much smaller bend in the helix at the Pro 14 residue, which is accommodated in part by a displacement of the Gly 11 carbonyl away from the helix axis.

The solvent accessibility³⁰ was calculated for each isolated alamethicin molecule without neighbouring peptide or solvent molecules. The solvent-accessible surface area is generally less than 5 Å² for carbonyl oxygen atoms participating in backbone hydrogen bonds. The carbonyls of Aib 10 and Gly 11 do not participate in backbone hydrogen bonds and have solvent accessibilities between 5 Å² and 17 Å². The carbonyl oxygen atoms fully exposed at the C-terminus of the molecules have calculated areas of 18–30 Å². The carbonyl groups of Aib 10 and Gly 11 of molecule I are hydrogen-bonded to methanol solvent molecules, while the carbonyl of Gly 11 of molecule III is hydrogen-bonded to the amide side chain of Gln 7 from molecule II. The carbonyls of residues 10 and 11 of molecule II and 10 of molecule III do not form hydrogen bonds with the solvent structure as it is presently modelled.

The alamethicin sequence contains four residues having polar side chain groups: Gln 7, Glu 18, Gln 19 and Phe 20. The Phe 20 hydroxyl group hydrogen bonds to the backbone in two of the three independent molecules. The carbonyl oxygen atoms of Aib 10 and Gly 11 are solvent-accessible and must also be considered as polar regions on the alamethicin molecular surface. Although the backbone structure differs among the three independent molecules in the C-terminal region, the distribution of polar groups on the surface of each molecule is very similar. The Gln 7 and Glu 18 side chains and the carbonyl oxygen atoms of Aib 10 and Gly 11 all lie on a narrow strip on the surface of each molecule parallel to the helix axis. The polar strip lies on the convex surface of the bent helices. The

Fig. 4 *a*, Schematic and computer graphics representations of the proposed pore. The alamethicin monomer as seen in the crystal is shown as two cylindrical segments signifying the interruption of the α -helical hydrogen bonding introduced by the Pro 14 residue. The stippled spheres at the mouth of the channel represent the Glu 18 residues while the open spheres at the centre and top of the channel structure indicate the hydrogen-bonded annulus of Gln 7 and Gln 19 residues, respectively. The parallel cylinders represent the N-terminal domain of the alamethicin molecules. The black dots on the face of each cylinder represent the solvent-accessible carbonyl groups of Aib 10 and Gly 11. The upper cork-shaped elements represent the C-terminal domains, and are tipped away from the channel n -fold axis, illustrating the bend imposed by the Pro 14 residue. The C-terminal elements are flaired to indicate the increasing bulk of the side chains in this region, maintaining van der Waals' contact even as the helix axes diverge. The stippled spheres represent the Glu 18 side chains, which are removed from direct contact due to the flair of the C-terminal region. The Gln 19 side chains are represented by the spheres at the top of the C-terminal segments illustrating the hydrogen bond interactions with the carbonyl oxygen atoms of a neighbouring molecule. *b*, A solvent-accessible surface representation of the complete alamethicin channel exterior, illustrating the strong surface complementarity of the hydrophobic exterior surface of the N-terminal region. *c*, A cutaway view of the alamethicin channel as in *a*, representing the solvent-accessible surface of each molecule. Alternate molecules are shaded light and dark to illustrate the intermolecular packing; the lighter shading represents solvent-accessible oxygen and nitrogen atoms of the backbone and side chains. Molecule I was used to form the channel structure without modification, thus modifications to the C-terminal regions of the molecule which would allow Gln 19 hydrogen bonding to neighbouring molecules have not been made. Also, the Gln 7 annulus does not seem as restricted as was observed in the CPK modelling, as the side chain geometry has not been altered to agree with the physical model. *d*, A view down the oligomer axis from the C-terminal surface.



Gln 19 side chain does not lie on this polar strip. Thus, the alamethicin structure is amphiphilic in two respects, as most of the polar groups are segregated in the C-terminal region of the primary structure, and lie along a narrow polar strip parallel to the helix axis in the tertiary structure.

The orientation of the three independent alamethicin molecules does not result in a symmetric trimer structure, but the independent molecules pack with each other and with molecules related by 2_1 -symmetry operations and translations to form a complex array of irregular channels, parallel to the x -axis of the unit cell. Although the alamethicin molecules pack to form solvent-filled channels, the polar residues do not segregate towards the centre of the channel as might be expected for a membrane ion channel. In any single solvent channel, both faces of a single molecule type contribute to the channel surface. The three independent molecules, identified as the asymmetric unit, are oriented in parallel with their C-termini close to the origin and their N-termini directed in the $+x$ direction, perpendicular to the 2_1 -symmetry axis. Molecules of the asymmetric unit pack in an antiparallel arrangement with symmetry-related molecules.

Construction of alamethicin channel models

For the construction of models of the alamethicin pore, a 'mean' monomer unit was used, representing essentially an average of the three peptide conformations observed in the crystal structure. The alamethicin conformation was idealized as two regular α -helical segments (1–13 and 14–20) with an angle of 20° between the two helix axes. A single $4 \rightarrow 1$ hydrogen bond (Val 15 to Leu 12) was introduced as seen in each of the monomers in the crystal. Such monomer units were assembled into oligomers using the following rules: (1) n -fold symmetry around the membrane plane normal, (2) asymmetric distribution between the two sides of the membrane, (3) optimization of side-chain packing between monomer units, (4) maximization of intermolecular hydrogen bonding. Two alamethicin CPK models were docked by hand in various orientations and the fit was assessed subjectively. One set of orientations resulted in a particularly favourable packing of side chains in the N-terminal domain; in this orientation the helix axis of the N-terminal segments is parallel to the oligomer axis. The relative rotation about the helix axis is such that the Gln 7 amide groups from neighbouring molecules form hydrogen bonds with good geometry. When such interactions are propagated, complete channels containing 8–12 molecules are generated. The most favourable oligomer size cannot be determined from these model-building studies, however the hydration structure described below favours an octameric channel structure.

The side chains of the N-terminal domains interdigitate to form a zipper-like structure. All side chains participating in this interface are short (Ala or Aib), consisting of only β -carbon atoms. The Pro 14 residue is directed towards the centre of the channel, causing the helix axis of each molecule to bend away from the oligomer symmetry axis (Fig. 4). The molecules still maintain van der Waals' contact in the C-terminal domain interface but with less favourable surface complementarity, as the side chains in this region are bulkier. The channel model has a funnel-like appearance with a flair at the C-terminal domain region, due to the bend in the helix axis induced by the Pro 14 side chain.

The interior of the channel contains three bands of polar groups capable of hydrogen bonding to water. Each Gln 7 residue donates and receives a Gln 7 hydrogen bond involving residues of each neighbouring molecule, forming a hydrogen-bonded annulus within the channel (Fig. 4). The amide plane of each Gln 7 side chain is tipped out of the mean plane of the Gln 7 residues; each amide is tipped in a direction opposite to that of its neighbours, resulting in a zig-zag pattern. The resulting structure projects amide hydrogen bond donors from each Gln 7 side chain towards the oligomer symmetry axis such that the amide protons are alternately directed above and below the mean Gln 7 plane.

The Gln 7 side chains are in a relatively low energy conformation while making two hydrogen bonds with good geometry. Such considerations indicate that the hydrogen-bonded annular structure will be relatively stable even in the presence of channel water. The unhydrated Gln 7 annulus produces a $7 \text{ \AA}$ diameter constriction in the octameric channel.

The carbonyl groups of Aib 10 and Gly 11 are solvent-accessible in the model as found for isolated molecules in the crystal. Such hydrogen bond acceptors will provide a further means of solvating the channel interior.

The Glu 18 side chains are also directed towards the oligomer symmetry axis, but because the helix axes of the monomers are directed away from the oligomer axis in this region of the molecule, they do not make direct contact. Indeed, the oxygen atoms of neighbouring carboxyl groups are separated by a minimum distance of $\sim 3.6 \text{ \AA}$. The Glu 18 carboxyl groups can be further separated if strict n -fold symmetry is not obeyed. In an aqueous salt solution at neutral pH, the Glu 18 side chain carboxyl groups would be ionized but would be sufficiently shielded from each other by a cloud of condensed counterions so that they would not greatly destabilize the channel structure.

The Gln 19 side chain is tangential to the C-terminal rim of the oligomer, such that the carbonyl is directed towards the oligomer axis while the two amide protons hydrogen bond to oxygen atoms of a neighbouring molecule. The hydrogen bonds are made to the hydroxyl of Phe 20 (which in turn is hydrogen-bonded to the carbonyl oxygen of Aib 17 and to the carbonyl oxygen of Glu 18). This hydrogen-bonding system continues throughout the oligomer structure, stabilizing the C-terminal domains and in part counteracting the residual electrostatic repulsion of the Glu 18 residues. The stabilization realized from the Gln 19 interactions will depend on the hydrophobicity of that region of the membrane.

The exterior of the ion channel is highly hydrophobic, containing many small alkyl side chains and no polar side chains.

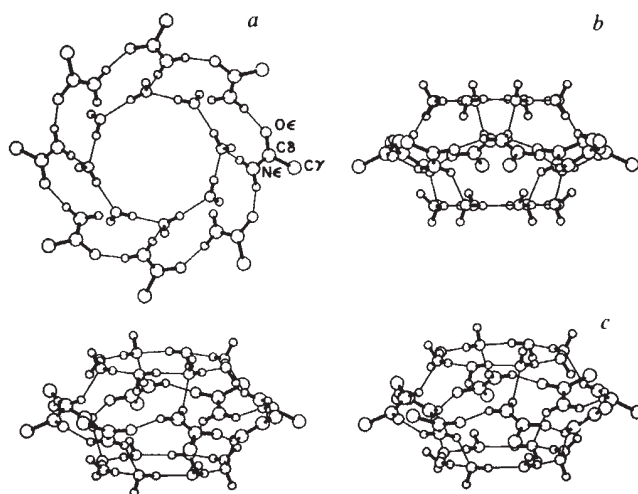
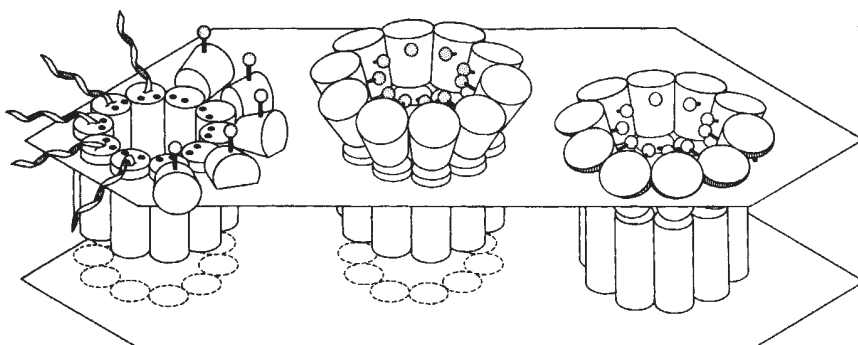


Fig. 5 Ball-and-stick drawings of one possible structure of the solvent near the Gln 7 annulus. *a*, The hydrated Gln 7 annulus viewed along the axis of the octameric channel model. The atoms of one side chain are labelled. Only atoms beyond C^β are shown. The outer ring hydrogen bonds connect amide and carbonyl groups in adjacent Gln residues; the inner ring of hydrogen bonds connects eight water molecules to each other. The roughly radial bonds connect the unused proton of alternating amide groups to four of the water oxygen atoms. For clarity, the lower ring and interstitial water molecules are not shown. *b*, The hydration structure of the Gln 7 annulus perpendicular to the oligomer axis. The alternating orientation of the Gln 7 side chains can be seen together with the hydrogen bonding to both rings of water. *c*, A stereo diagram of the hydrated Gln 7 annulus. Four additional water molecules can easily be added inside the cage with many hydrogen-bonding possibilities. The mass density of such a 14-molecule hydration complex would be close to that of bulk water.

Fig. 6 Voltage-gating channel model. The parallel planes specify the boundary of the alkyl chain region of a lipid bilayer. Elements of the channel model which are in common with Fig. 4a are as described in legend. The left-hand channel illustrates the structures proposed for the alamethicin oligomer in the absence of an applied voltage. The helical ribbons signify an extended random coil structure. The cork-shaped elements represent the C-terminal region of each molecule, bent away from the channel n -fold axis and partially buried in the alkyl chain region. The channel model in the centre represents a hypothetical intermediate produced by an applied voltage. The model on the right is proposed as the open form of the ion channel.



The conformation of the model used in the construction of the oligomer contains only one non-hydrogen-bonded amide within the central region of the α -helix; this amide is located on the exterior of the channel.

The length of the channel along the oligomer axis is 32 Å, which is about the thickness of the alkyl chain region of biological and black lipid membranes. Thus, each end of the alamethicin molecule will be at the interface of the head group and alkyl chain regions of the lipid bilayer.

The distribution of polar groups in the alamethicin channel is much more regular than that found in globular proteins, as the polar groups within the channel occur in bands at different elevations. The first hydration layer within the channel must interact with this regular array of polar groups in a limited number of orientations, leading to a hydration structure which may be much more regular and extensive than that normally found surrounding globular proteins in crystals or in solution.

Ordered water structure may be of functional significance in the region occupied by the Gln 7 residues. The Gln 7 annulus is the most restricted region of the channel and contains a regular array of hydrogen bond donors directed towards the oligomer axis. The details of the hydration structure will depend on the number of monomers in the alamethicin oligomer. In general, each Gln 7 side chain amide proton will donate a hydrogen bond to a water oxygen atom, causing a further constriction in the ion channel. Such hydrogen-bonded water molecules would be further stabilized by a second set of water molecules bridging the first hydration layer, resulting in a hydrogen-bonded ring of water above and below the Gln-7 annulus. The hydrated Gln 7 annulus for an octameric channel is illustrated in Fig. 5. The diameter of the hydrated annulus region accessible to bulk solvent molecules or ions is ~4.5 Å for the octamer.

Functional implications of the channel model

We suggest that changes in the size or geometry of the Gln-7 annulus are responsible for the conductance substates observed in single alamethicin channels, as this structure provides the greatest restriction to the alamethicin channel cross-section. The model-building studies have not led to a unique channel structure, but to a family of oligomer models containing 8–12 monomers. Thus, the barrel-stave model^{14,18} cannot be excluded by model-building studies alone. A channel oligomer of fixed composition could give rise to the conductance substate fluctuations if the stability of the Gln 7 annulus is altered by the applied voltage. The dipole moment of each Gln 7 side chain amide group is oriented almost perpendicular to the electric field in the channel model presented above. If the electric field is sufficient, the amide group will reorient such that the carbonyl is directed towards the C-terminus of the channel. Such a reorientation would result in an enlargement of the channel diameter while exposing a Gln 7 carbonyl group which could interact favourably with a hydrated cation, thus providing a path for cation movement through the channel. Such a reorientation would disrupt the hydration structure, which would also lead to an increase in the channel cross-

section. The involvement of different numbers of amide groups in such reorientation might explain the fluctuating conductance substates, although persistence for milliseconds implies the existence of activation barriers whose structural origin is not yet clear.

A molecular explanation of the voltage-gating event requires an understanding of the alamethicin molecular structure in the resting membrane and the physical basis for the voltage-driven structural change. A physical model for the voltage-gating process is presented in Fig. 6. We suggest that the hydrophobic N-terminal segment of alamethicin (residues 1–10) inserts into the alkyl chain region of the lipid bilayer without the aid of an applied voltage, forming a solvent-filled channel traversing one-half of the alkyl chain region. This preferential insertion is expected because of the small number of solvent-accessible polar backbone and side chain atoms present in that segment relative to the more hydrophilic C-terminal region. The insertion of the N-terminus of the alamethicin α -helix into the lipid bilayer results in a favourable orientation of the molecular dipole moment in the electric field generated when that side of the membrane is at a positive potential. The C-terminal portion of the molecule is modelled either as a random coil, associating with the head group region of the bilayer or projecting into the solvent, or as an α -helical segment bent away from the channel n -fold axis. The lack of hydrogen bonds to the carbonyl groups of residues 10 and 11 (Fig. 3) which project towards the channel n -fold axis, would allow such a hinge motion without disrupting any hydrogen bonds observed in the crystal conformers or as proposed in the model, and would allow the hydrophobic exterior surface of the C-terminal segment to partition into the alkyl chain region of the bilayer. The dipole moment of the C-terminal helical segment would be perpendicular to the electric field direction in the bent helix model, while the dipole moments of the individual peptide groups would be randomly oriented if the C-terminal segment were in a random coil conformation.

An electric field applied to the alamethicin molecules in the sense used in conductance experiments would favour either a coil-helix transition or a reorientation of the C-terminal helical segment, both resulting in the intermediate shown in the centre of Fig. 6. This C-terminal exterior surface now presents a larger hydrophobic surface to the head group and aqueous phase region of the membrane. The burial of this surface in the alkyl chain region of the bilayer would thus serve as the driving force for the voltage-gating event. The inserted alamethicin channel is of sufficient length to span the alkyl chain region of the bilayer but would not extend significantly into the head group region.

Recently, two results have been published which are consistent with the proposed structure of the ungated alamethicin oligomer illustrated in Fig. 6: (1) cross-linking of a lipid photo-label to alamethicin in liposomes results in labelling of only the N-terminal segment (residues 1–13), indicating that this portion of the peptide is accessible to the alkyl chain region of the bilayer³¹; and (2) electron micrographs of negatively-stained liposomes containing alamethicin reveal a circular stain-excluding structure which is stained at its centre³².

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1. Mueller, P. & Rudin, D. O. *Nature* **217**, 713-719 (1968).
2. Payne, J. W., Jakes, R. & Hartley, B. S. *Biochem. J.* **117**, 757-766 (1970).
3. Balasubramanian, T. M. *et al. J. Am. chem. Soc.* **103**, 6127-6132 (1981).
4. Gisin, B. F., Kobayashi, S., Davis, D. G. & Hall, J. E. in *Proc. 5th Am. Peptide Symp.* (eds Goodman, M. & Meienhofer, J.) 215-218 (Wiley, New York, 1977).
5. Pandey, R. C., Cook, J. C. & Rinehart, K. L. *J. Am. chem. Soc.* **99**, 8469-8483 (1977).
6. Ovchinnikov, U., Kiryushkin, A. & Kozhevnikova, I. V. *J. gen. Chem. U.S.S.R.* **41**, 2105-2116 (1971).
7. Martin, D. R. & Williams, R. J. P. *Biochem. J.* **153**, 181-190 (1976).
8. Lattore, R. & Alvarez, O. *Physiol. Rev.* **61**, 77-150 (1981).
9. Eisenberg, M., Hall, J. E. & Mead, C. A. *J. Membrane Biol.* **14**, 143-176 (1973).
10. Gordon, L. G. M. & Hayden, D. A. *Phil. Trans. R. Soc. B270*, 433-447 (1975).
11. Boheim, G. & Kolb, H. A. *J. Membrane Biol.* **38**, 99-150 (1978).
12. Latorre, R. & Donovan, J. J. *Acta physiol. scand. Suppl.* **481**, 37-45 (1980).
13. Gordon, L. G. M. & Hayden, D. A. *Biochim. biophys. Acta* **255**, 1014-1018 (1972).
14. Boheim, G. *J. Membrane Biol.* **19**, 277-303 (1974).
15. Gordon, L. G. M. in *Drug and Transport Processes* (ed. Callingham, B. A.), 251-264 (Baltimore University Press, 1974).

16. Eisenberg, M., Kleinberg, M. E. & Shaper, J. H. *Ann. N.Y. Acad. Sci.* **303**, 281-291 (1977).
17. Hanke, W. & Boheim, G. *Biochim. biophys. Acta* **596**, 456-462 (1980).
18. Bauman, G. & Mueller, P. *J. supramolec. Struct.* **2**, 538-557 (1974).
19. Hall, J. E. *Biophys. J.* **15**, 934-939 (1975).
20. McMullen, A. I., Marlborough, D. I. & Bayley, P. M. *FEBS Lett.* **16**, 278-280 (1971).
21. Jung, G., Dubishar, N. & Leibfritz, D. *Eur. J. Biochem.* **54**, 395-409 (1975).
22. Shamala, N., Nagaraj, R. & Balaram, P. *Biochem. biophys. Res. Commun.* **79**, 292-298 (1977).
23. Smith, G. C. *et al. J. Am. chem. Soc.* **103**, 1493-1501 (1981).
24. Butters, T. *et al. Angew. Chem.* **93**, 904-905 (1981).
25. Meyer, C. E. & Reusser, F. *Experientia* **23**, 85-86 (1967).
26. Sowadski, J. M., Foster, B. A. & Wyckoff, H. W. *J. molec. Biol.* **150**, 245-272 (1981).
27. Blow, D. M. & Crick, F. H. C. *Acta crystallogr.* **12**, 749-802 (1958).
28. Hendrickson, W. A. & Konnert, J. H. in *Biomolecular Structure Function, Conformation and Evolution* (ed. Srinivasan, R.) 43-57 (Pergamon, Oxford, 1981).
29. Connolly, M. thesis, Univ. California (1981).
30. Lee, B. K. & Richards, F. M. *J. molec. Biol.* **55**, 379-400 (1971).
31. Latorre, R., Miller, C. G. & Quay, S. *Biophys. J.* **36**, 803-809 (1981).
32. McIntosh, T. J., Ting-Beall, H. P. & Zampighi, G. *Biochim. biophys. Acta* **685**, 51-60 (1982).

The sequences of an expressed rat α -tubulin gene and a pseudogene with an inserted repetitive element

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The rat genome contains two segments closely related to a rat α -tubulin mRNA. Both have been cloned and complete nucleotide sequences are presented. Analysis of the structure and sequence of one of these establishes it as a functional α -tubulin gene. The second segment is a processed α -tubulin pseudogene. Comparison of this pseudogene to the mRNA and gene coding for α -tubulin strongly suggests that a mature mRNA was involved in its origin. Features of the pseudogene and a dispersed repetitive element inserted within it possibly reflect a common RNA-mediated process of insertion.

MICROTUBULES are ubiquitous cellular structures found in eukaryotic organisms and responsible for a variety of functions. The basic subunit of the microtubule is a heterodimer composed of two proteins, the α - and β -tubulins. The amino acid sequence of each of these proteins is conserved and their gene sequences cross-hybridize among distantly related organisms¹. The multiple functions of microtubules might reflect multiple tubulin proteins with different amino acid sequences and thus multiple genes. Amino acid sequence² and nucleotide sequence data^{3,4} have shown that mammalian α -tubulin protein is microheterogeneous within species. Whether this microheterogeneity reflects functionally specific α -tubulin proteins is not known.

All tubulin genes are probably derived from a common ancestral sequence. Analysis of the number of α - and β -tubulin genes in mammals using cDNA sequences as probes has detected 10-20 copies of each per genome⁵⁻⁷. These segments could either represent functional genes or nonfunctional pseudogenes. On the basis of their sequence organization, pseudogenes can be divided into two classes: simple duplications of the expressed loci with subsequent divergence of nucleotide sequences, or copying of sequences present in RNAs and integration of these cDNA-like segments. The latter type has been referred to as processed pseudogenes⁸.

It has been proposed that pseudogenes of small nuclear RNAs were created through RNA intermediates⁹. These pseudogenes are typically flanked by short direct repeats that are thought to have been generated during integration of the pseudogenes. Many dispersed repetitive elements in mammalian DNA, such as human Alu elements, are also flanked by short direct repeats, perhaps also created by their insertion (reviewed in ref. 10).

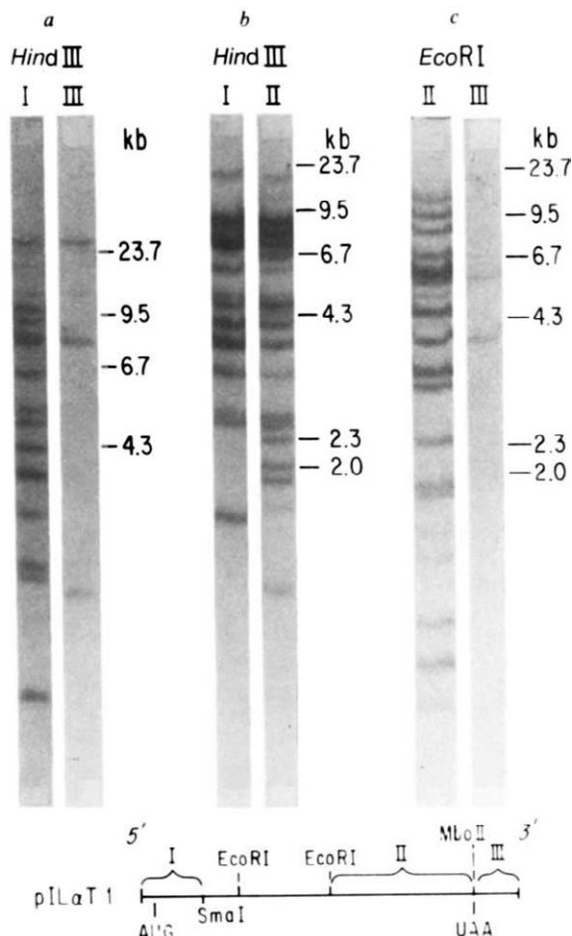
The similarity in structure among processed pseudogenes, pseudogenes of small nuclear RNA and Alu dispersed repetitive elements suggests that these all could result from a common process involving RNA. We report the sequence of an expressed rat α -tubulin gene, a processed pseudogene derived from it and a rat dispersed repetitive element inserted into this pseudogene. The structures of these elements are consistent with the hypothesis that the pseudogene and the dispersed repetitive element were created through RNA intermediates.

Cloning of rat DNA segments closely related to rat α -tubulin mRNA

The sequence of a cDNA clone of rat brain α -tubulin mRNA has been described previously⁴. The cDNA clone pLaT1 is 1,617 nucleotides in length and possesses sequences from the 3' poly(A) tract, through the coding sequences to within approximately 33 nucleotides of the 5' terminus of an abundant α -tubulin mRNA. Twenty-nine of these 33 nucleotides were determined by sequencing primer extension products⁴; the total sequence is shown in Fig. 3.

Others have detected multiple copies of genomic α -tubulin sequences in a variety of species¹¹⁻¹⁴. To determine the number of α -tubulin genes in rat, three probes were prepared from the α -tubulin cDNA clone; probes I and II encompass sequences of the amino and carboxyl terminal portion of α -tubulin, respectively, while probe III contains sequences totally included within the 3'-untranslated portion of the mRNA. As α -tubulin is a highly conserved protein, probes I and II should hybridize to most of the α -tubulin related sequences in the rat genome.

Fig. 1 Genomic representation of different portions of α -tubulin cDNA. Sprague-Dawley rat liver genomic DNA was extracted³⁷ and cleaved to completion by either *Hind*III or *Eco*RI endonucleases, as indicated above each set of lanes. *Hind*III does not cleave the cDNA pIL α T1, while *Eco*RI cleaves the cDNA at two positions. The spectrum of fragments was resolved by electrophoresis (10 μ g per lane) and transferred to nitrocellulose³⁸. cDNA probes were prepared by digestion of pIL α T1⁴ with the indicated restriction endonucleases and isolation of appropriate fragments from preparative agarose gels by the sodium iodide-glass bead method³⁹. ³²P-labelling was performed by nick-translation⁴⁰. Hybridization and washing (see below) of blots was done essentially as described previously⁴. Three different probes were used: probe I encompassing 66 nucleotides of the 5' untranslated (UT) and 190 nucleotides of the amino-terminus portion of α -tubulin mRNA, probe II consisting of 590 nucleotides of COOH-terminal information and ending 5 nucleotides before the termination codon and probe III, totally contained in the 3'-UT portion of the mRNA. *a*, probe I hybridizes to 16 fragments. All of the 16 bands persist through stringent washes (0.5–2 $\times$ SSC, 65 °C). Hybridization of genomic blots with probe II (*b* and *c*) allows an estimate of 14 copies of sequences homologous to the carboxyl-terminal portion of α -tubulin mRNA. In total, 10 fragments hybridize to both probes I and II (part B). The lane in *a* probed with III and washed at a moderate stringency (2 $\times$ SSC, 65 °C) contains four different fragments homologous to the 3'-UT portion of α -tubulin mRNA. However, in *c* only two fragments in an *Eco*RI digest remain hybridized after a high stringency wash (0.2 $\times$ SSC, 65 °C). Therefore, the rat genome contains only two copies of α -tubulin 3'-untranslated sequences closely related to the mRNA represented in pIL α T1.



Sequences in the 3'-untranslated region of the mRNA should be less constrained and could perhaps be used to distinguish between rat genomic sequences closely related to the cDNA clone and those more distantly related. This is in fact the case. The amino-terminal and carboxyl-terminal probes hybridize to 16 and 14 *Hind*III fragments of rat DNA, respectively (see Fig. 1a, b). Ten of these bands hybridize to both probes, suggesting that these fragments contain the entire α -tubulin coding sequence. However, only two *Eco*RI bands hybridize in stringent conditions to the probe from the 3'-untranslated portion of the mRNA, suggesting that there are only two genes closely related to the cDNA clone (see Fig. 1c).

λ -Phage libraries of rat liver DNA sequences were screened by the method of Benton and Davis¹⁵ with radioactive probes from both the 5'- and 3'-untranslated (UT) portion of the cDNA clone pIL α T1. We had shown previously that these two probes

hybridize in stringent conditions to the same set of genomic bands in a Southern blot. Seven phage plaques detected as positive with both probes in the first screen yielded positive hybridization signals in subsequent screening and these independent phages were selected for further study. Southern blotting of restriction endonuclease digests of the phage DNAs distinguished two phages, $\lambda\alpha$ T15 and $\lambda\alpha$ T14, that hybridized to the probes under high stringency. These two phages clearly had different restriction cleavage patterns.

Mapping of α -tubulin sequences within recombinant phages

Electron microscopy was used to map sequences complementary to α -tubulin mRNA in both $\lambda\alpha$ T15 and $\lambda\alpha$ T14. First, hybridization and R-loop mapping showed that both phage

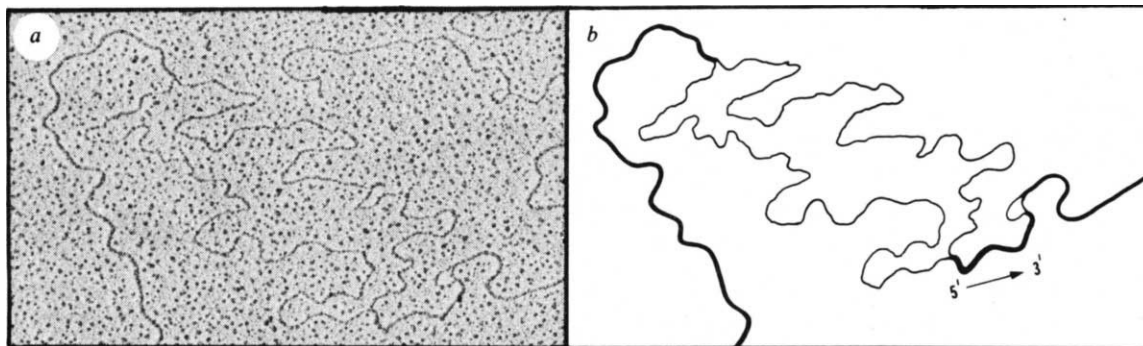


Fig. 2 Electron microscopic analysis of recombinant phage DNA. A heteroduplex formed between $\lambda\alpha$ T14 and $\lambda\alpha$ T15 DNAs is shown in *a* and represented diagrammatically in *b*. Hybrids were formed and mounted as described⁴¹ with minor modifications. A several-hundred-nucleotide region of non-homology separates a 1,590 $\pm$ 110-nucleotide duplex segment from the long arm of the Charon 4A vector DNA. The position of this double-stranded region with respect to the positions of R-loops (formed by hybridization of mRNA and phage DNA; not shown) and with respect to restriction and blotting data established this as the segment homologous to α -tubulin cDNA. This combination of data also allowed the assignment of the designated polarity with respect to α -tubulin mRNA sequences.

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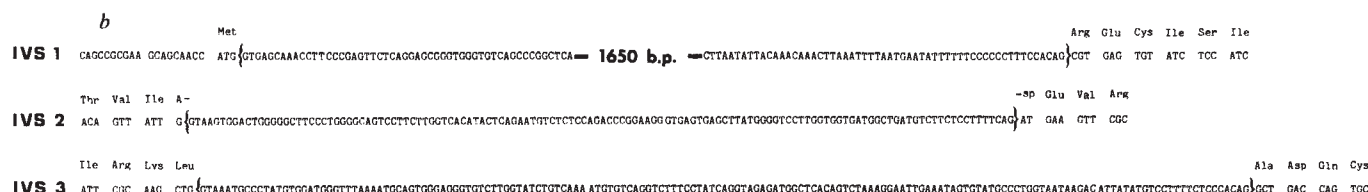


Fig. 3 Sequence of α -tubulin gene and pseudogene. **a**, The sequence of the complete α -tubulin mRNA as determined previously⁴ is presented along with the sequence of the α -tubulin gene ($\lambda\alpha$ T14) in the correct alignment above the mRNA. Sequences upstream of the mRNA initiation nucleotide (+1) are numbered beginning at -1. Putative transcriptional regulatory regions, GTATATAAGG at -31 and GGACCAA at -77 are boxed. The ATG initiation codon is underlined. Positions of intervening sequences are denoted (IVS1-3). The α -tubulin mRNA is polyadenylated at one of four terminal residues (overlined and labelled poly A). Note that the coding sequence of the gene is identical to that of the mRNA. The sequence of the α -tubulin processed pseudogene ($\lambda\alpha$ T15) is aligned below the sequence of the mRNA. Comparison with the gene reveals close homology up to the mRNA initiation nucleotide (+1) and no significant homology further upstream (mismatches are indicated by asterisks). Several small deletions and two single base insertions into the α -tubulin mRNA homologous sequences in $\lambda\alpha$ T15 are represented by underlined gaps or arrows, respectively. All three intervening sequences are precisely absent. The $\lambda\alpha$ T15 sequences homologous to α -tubulin mRNA are immediately flanked by short 14-nucleotide direct repeats of AAAAAGAGATTTT (underlined and labelled 5' or 3' D.R.). Note that comparison of 3'-flanking sequences in $\lambda\alpha$ T14 and $\lambda\alpha$ T15 reveals no significant homology. The position of the inserted repetitive element (R.dre.1) found in the pseudogene is shown by an arrow. This element is flanked by direct repeats of 15 nucleotides which occur once in α -tubulin mRNA. These 15 nucleotides are underlined. The sequence of this inserted element and direct repeats is shown in Fig. 4. **b**, Positions and sequences of introns found in the α -tubulin gene ($\lambda\alpha$ T14). Sequences of introns are presented with surrounding coding regions. The introns are bracketed. Approximately 60% of the large intron IVS1 sequence has been determined.

DNAs contained sequences complementary to mRNA extracted from rat neuroblastoma cells (line B50; not shown). The RNA/DNA duplex arm of the R-loop measured $1,510 \pm 130$ nucleotides in length, the same approximate length as the α -tubulin mRNA. Second, the R-loop structures did not reveal the presence of long introns in either duplex. However, in $\lambda\alpha$ T14 we frequently observed a cross-over of the two arms of the R-loop at a site ~ 540 nucleotides from one end. This would be consistent with the presence of a short intron.

Heteroduplex analysis of $\lambda\alpha$ T14 and $\lambda\alpha$ T15 showed that the two inserted rat DNA segments contained $1,590 \pm 110$ nucleotides of sequence homology, just sufficient to encompass the total α -tubulin mRNA sequences (Fig. 2a). The homologous sequences were not interrupted by long segments of non-homologous DNA and were flanked by single-stranded loops which did not hybridize to one another in these mounting conditions. In the heteroduplexes of $\lambda\alpha$ T14 and $\lambda\alpha$ T15 two small knobs were observed at reproducible positions along the duplex segments. Similar knobs have previously been observed for small deletions or insertions in heteroduplexes and suggest that the region of homology is interrupted by a few short segments of nonhomologous sequence. In 5% of the heteroduplexes a single-strand loop of about 1,500 nucleotides was formed at one end of the duplex, suggesting the presence of a larger intron.

Restriction endonuclease cleavage maps were prepared for both $\lambda\alpha$ T14 and $\lambda\alpha$ T15 phage DNAs by Southern blot analysis using probes similar to those described for Fig. 1. This mapped the α -tubulin related sequences in the phage DNAs to positions adjacent to the longer arm of the Charon 4A vector, and oriented these sequences as indicated in Fig. 2b.

DNA sequence of a rat α -tubulin gene

The nucleotide sequence of the α -tubulin related portion of both $\lambda\alpha$ T14 and $\lambda\alpha$ T15 recombinants was determined by the chemical cleavage method of Maxam and Gilbert¹⁶. Initially DNA sequencing was done by directly labelling termini in recombinant phage DNA. Subsequently, portions of the α -tubulin related sequences in both $\lambda\alpha$ T14 and $\lambda\alpha$ T15 DNA were subcloned into the plasmid pBR325 for further sequencing. In total, $\sim 6,000$ nucleotides of sequence were determined and over 80% was read from either both strands or from one strand terminally labelled at different cleavage sites.

Recombinant $\lambda\alpha$ T14 probably contains sequences specifying an expressed α -tubulin gene of the rat. The nucleotide sequence of this gene is compared with that of the previously sequenced rat α -tubulin cDNA, pIL α T1, in Fig. 3a. All of the rat α -tubulin mRNA sequence is found in $\lambda\alpha$ T14 but is interrupted by three introns. In Fig. 3a the positions of the introns are denoted and the sequence of part or all of each intron is given in Fig. 3b.

All three introns have consensus splicing sequences at their 5' and 3' boundaries, permitting assignment of their position in the coding sequence of α -tubulin¹⁷. Twenty-eight nucleotides upstream from sequences complementary to the suggested 5' end of the α -tubulin mRNA, is the sequence TATAA-, the expected TATA or Goldberg-Hogness sequence¹⁸. In preliminary experiments, this DNA segment has been tested for transcription template activity in the whole cell extract system¹⁹. The length of RNA polymerase II runoff transcripts suggests that the *in vitro* system initiates RNA at a position near or identical to that of the 5' end of the mRNA, consistent with this being the initiation site of α -tubulin mRNA synthesis.

DNA sequence of a rat α -tubulin pseudogene

Recombinant $\lambda\alpha$ T15 contains an interesting α -tubulin pseudogene. First, the α -tubulin related sequences in this phage are remarkably similar to those of the cDNA clone, pIL α T1 (see Fig. 3a) and therefore to the α -tubulin mRNA. As in the mRNA, sequences corresponding to all three introns in the expressed gene have been precisely excised from the α -tubulin related sequences in $\lambda\alpha$ T15. In addition, this gene is homologous to sequences at the 5' and 3' termini of the α -tubulin mRNA but the homology to the expressed gene ends precisely at the limits of the mRNA. For example, sequences complementary to the suggested 5' end of α -tubulin mRNA are present in $\lambda\alpha$ T15 and $\lambda\alpha$ T14 but sequences immediately upstream from this site are totally different (shown in Fig. 3a). In the $\lambda\alpha$ T15 case, 14 nucleotides of sequence immediately 5' to the mRNA-related sequences are directly duplicated at the 3' boundary of the mRNA-related sequences. A short poly (dA) tract is present at the 3' terminus of the mRNA related sequences in $\lambda\alpha$ T15, immediately preceding and included in the second copy of the 14-nucleotide direct repeat. There is no significant homology between $\lambda\alpha$ T15 and $\lambda\alpha$ T14 DNAs downstream from this position. Some 140 and 540 nucleotides of sequence have been determined 5' and 3', respectively, of the α -tubulin related sequences in $\lambda\alpha$ T15 DNA.

DNA sequence of a rat dispersed repetitive element interrupting the rat α -tubulin pseudogene

The α -tubulin related sequences in the pseudogene are not completely colinear with α -tubulin cDNA; they are interrupted by a 110-nucleotide segment and a duplication of 15 nucleotides of tubulin sequence at position 1,119 (denoted in Fig. 3a). Figure 4 shows the sequence of the 110-nucleotide segment, and the flanking 15-nucleotide direct repeats. This sequence has several features common among dispersed repetitive elements, particularly the Alu-like elements of human, mouse and

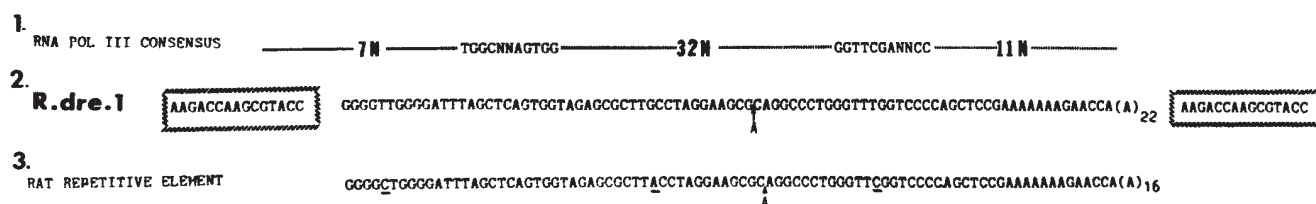


Fig. 4 Relationship of repetitive element (termed R.dre.1) contained in the α -tubulin pseudogene. Two-dimensional matrices were used to align the sequence of R.dre.1 with sequences of a closely related element found as the third member of a tandem array of three repetitive sequences located in an intron of rat growth hormone²¹. A second such element (not shown), found several kilobase pairs upstream from the α -tubulin pseudogene, shows a comparable relatedness to R.dre.1. Similar alignments were attempted with consensus sequences for human Alu-like elements, rat 4.5S RNA and rat 7S RNA⁴² with only marginal success. These elements are not closely related to R.dre.1. The top line of the figure shows the consensus RNA polIII promoter elements²⁰ derived from a compilation of tRNA sequences and present in all DNAs thought to be transcribed by this polymerase. In all the comparisons mismatches are underlined, deletions are represented by (–) and insertions are designated.

Chinese hamster DNAs¹⁰. The 5' portion of the 110-nucleotide segment contains a consensus RNA polymerase III initiation sequence²⁰ while the 3' portion has the customary A-rich sequence. A specific probe for this 110-nucleotide segment hybridizes as a smear to a Southern blot of an *Eco*RI digest of rat DNA. In addition, partial sequence data have revealed the presence of another such element upstream from the pseudogene in λ T15. Barta *et al.*²¹ have reported the sequence of an array of repetitive elements in an intron of a rat growth hormone gene. These repetitive elements occur as a tandem array of three related segments. The sequence of one component of this array is also shown in Fig. 4 and only differs in three positions from the element in the rat α -tubulin pseudogene. Clearly, the 110-nucleotide segment, designated R.dre.1 (rat dispersed repetitive element 1), is a member of a rat dispersed repetitive element family and is structurally related to the Alu-like elements.

Discussion

The sequence reported here from λ T14 is the only example of a fully characterized α -tubulin gene which is expressed. We propose that this is a functional gene because: (1) its exon sequences are identical to those of the previously reported α -tubulin cDNA and (2) the only other closely homologous 3'-untranslated sequence in rat is part of an α -tubulin pseudogene. It is likely that there are at least two α -tubulin genes expressed in rat cells, as a second partial cDNA sequence has been reported³. However, *S*₁ nuclease digestions of RNA/DNA hybrids have shown that the α -tubulin gene described here accounts for a large fraction of the α -tubulin mRNA in rat brain as well as in neuroblastoma, fibroblast and glioma cells in culture (I.L. and P.A.S., unpublished observations).

The active rat α -tubulin gene contains three introns, all begin and end in the consensus splicing sequences¹⁷ GT-----CAG. The first intron of ~1,770 nucleotides interrupts the coding sequence after the initiation codon ATG, is a class 0 intron¹⁷ and delineates a protein domain of one amino acid. The second intron of 130 nucleotides interrupts codon 76 after the first nucleotide and is thus a class 1 intron. The third intron of 165 nucleotides falls between codons 125 and 126 and is thus another class 0 intron. In view of the limited information about the tertiary structure of tubulin, it is not possible to relate gene structure with protein structure.

Two human DNA segments containing α -tubulin related sequences have been characterized by electron microscopy⁵. It is not clear whether these human DNA segments are expressed α -tubulin genes. These segments hybridize to a chicken cDNA segment but not to human cellular mRNA; both segments have two introns in roughly the same positions as those of the first two introns in the rat gene. However, one segment clearly has a third long intron in the carboxyl-terminal portion of the cDNA which is not present in this rat gene.

Tubulin is a ubiquitous eukaryotic protein and comparison of the number and positions of α -tubulin gene introns in species from different kingdoms might suggest when introns and RNA

splicing developed during evolution. A similar comparison has been partially made for actin genes with bewildering results. Intron positions in yeast and *Drosophila* actin genes are unique, while intron positions in sea urchin and vertebrates (both deuterostomes) are frequently conserved²². Interestingly, actin genes of vertebrates and soybean have an intron position in common, suggesting that the primordial gene contained this intron²². Whether the highly conserved α -tubulin genes will have a similar diversity of intron positions remains to be determined.

As α -tubulin is a major cellular protein, its transcription initiation site should be a strong constitutive promoter. The sequence around the promoter site conforms well with consensus sequences derived from comparison of other sites²³: GTATATAAGG is found at –31 and GGACCAA is positioned at –77. This DNA segment is active when added as template to a whole cell extract (I.L., unpublished results) but not very active. At the 3' end of the gene, polyadenylation occurs somewhere in a tract of 3A residues following the customary AATAAA²⁴.

The intracellular level of α -tubulin mRNA is apparently regulated by the concentration of free tubulin protein^{25,26}. The mechanism of this regulation whether transcription, RNA stability or RNA processing is not known. It should be possible to investigate this autoregulation of α -tubulin protein synthesis with probes prepared from the sequenced expressed gene. In addition, sequences responsible for α -tubulin mRNA synthesis and regulation can be defined by *in vitro* modification of the α -tubulin gene and transfection of cells.

The only other cellular DNA sequence highly homologous to the rat α -tubulin cDNA is a pseudogene. This pseudogene was undoubtedly formed by copying of a mature mRNA transcribed from the expressed α -tubulin gene. All sequences corresponding to introns have been precisely deleted, the pseudogene sequences commence at the 5' end of the mRNA and terminate at or in the poly(A) tract forming the 3' end of the mRNA. In addition, a direct repeat of 14 nucleotides flanks the precise 3' and 5' ends of the pseudogene. This structure strongly suggests that an α -tubulin mRNA was copied into DNA sequence and inserted into a cellular sequence creating the 14 nucleotide direct repeat. Since the pseudogene should be neutral in terms of genetic selection, and differs from the expressed gene by 38 point substitutions in 1,646 positions, the time of its creation can be estimated²⁷. Neutral mutations are fixed in the germ line of mammals at a rate of 0.7% per million years. Thus the 2.3% divergence of the pseudogene suggests that it arose 3–5 $\times 10^6$ yr ago in the germ line of rat. This is well after rat and mouse diverged (30 $\times 10^6$ yr)²⁸ and suggests that species even more closely related to rat may not contain this pseudogene.

Evidence for RNA intermediates in the generation of a subclass of pseudogenes has been previously obtained. Pseudogenes related to small nuclear RNAs are characterized by direct repeats that immediately bracket RNA related sequences⁹. Pseudogenes of α -globin of mouse²⁹ and human λ immunoglobulin⁸ are also thought to be RNA derived as they

lack introns and the latter terminates in a poly(A) tract. In these two cases, pseudogene sequences related to the expressed loci extend beyond the 5' terminus of the differentiated cell mRNA. This predicts that an aberrant transcript was used as template in generating these pseudogenes. Two (human) β -tubulin processed pseudogene sequences have also been described^{30,31}; each seems to span the amino acid coding sequences and terminates in a poly(A) tract. However, the structures of expressed β -tubulin genes are not known and limit further comparison. In all four cases, the pseudogene sequences are flanked by direct repeats.

A rat dispersed repetitive element is present in the α -tubulin pseudogene sequences. This element (R.dre.1) could have been inserted either in an expressed locus or after the RNA intermediate was used in the creation of the characterized pseudogene. In either case, it is likely that insertion of the repetitive element inactivated the gene and the total began evolving as a neutral sequence. Thus, R.dre.1 should have diverged from its progenitor sequences by less than 2.3%, the divergence of the pseudogene sequence in which it is found. Dispersed repetitive elements almost identical to R.dre.1 have been sequenced by others. An intron of rat growth hormone gene contains a 104-nucleotide segment which only differs in three positions from the repetitive element in the pseudogene (see Fig. 4)²¹. More interestingly, five members of a rat repetitive element family that hybridize to a brain-specific RNA of 160 nucleotides are similarly related to the R.dre.1 element³². The function of the 160-nucleotide brain-specific RNA is not known. Sequences related to R.dre.1 are also found in brain-specific hnRNA suggesting that their expression may be specific for certain differentiated cells. This has led to the proposal that these elements be called 'identifier sequences (ID)'³². Since the repetitive element in the pseudogene is mobile, any differentiated cell-specific transcription would suggest specific insertion into transcription units only expressed in that cell type. How this specificity could be recorded in germ cells is unclear. The fact that seven rat elements related to R.dre.1 are almost identical in sequence implies that this family of 100,000 dispersed elements is more highly conserved than most families of dispersed repetitive elements. Whether this reflects their recent creation or a high frequency of gene conversion remains to be determined. Given the short period of time that R.dre.1 has had to diverge in sequence, it is likely that these elements are very similar in sequence to that of the hypothetical template RNA. Identification of this template RNA is critical in advancing our understanding of these processes.

Dispersed repetitive elements have been most extensively studied in human DNA where 500,000 copies of a family of related sequences, 300 nucleotides in length, are found, the Alu family of dispersed repetitive elements¹⁰. About 80% of the members of this family are bracketed by direct repeats of

7–20 nucleotides. The hallmarks of Alu-like elements are nucleotide sequences characteristic of the RNA polymerase III consensus promoter in the 5' portion and sequences rich in poly(A) tracts in the 3' portion of the element¹⁰. Many of these elements are templates for *in vitro* transcription by RNA polymerase III³³. The RNA product is typically initiated at the 5' boundary of the repetitive element immediately downstream from the direct repeat. RNA polymerase III consensus sequences are clearly present in the repetitive element (R.dre.1) in the rat α -tubulin pseudogene (Fig. 4). A duplication of 15-nucleotides flanks R.dre.1 in the pseudogene. This duplication probably occurred during insertion of the repetitive element. A similar argument has previously been made for a direct repeat flanking a repetitive element in a monkey satellite sequence³⁴. Direct repeats at the site of insertion are characteristic of mobile elements such as transposons in bacteria, ty elements in yeast, copia elements in *Drosophila* and retroviruses in mammals³⁵. Generation of direct repeats of 7–20 nucleotides is not characteristic of integration of DNA fragments during DNA transformation of mammalian cells³⁶. This argues strongly for an active integration process in the mobility of Alu-like repetitive elements.

The striking parallels in the structure of the α -tubulin pseudogene and the rat repetitive element inserted within it, suggest that the two sequences were generated by the same process, a process that (1) copies RNA sequences into DNA sequences, (2) creates direct repeats on insertion, (3) retains the colinearity of inserted sequence and RNA intermediate, and (4) perhaps creates or requires an adenine-rich tract at the 3' terminus of the RNA or DNA intermediate. Given that there are 500,000 dispersed repetitive elements in human DNA, insertion of small repetitive elements in the germ line of mammals must not be uncommon. It seems reasonable to propose that pseudogenes of mRNAs are generated by the occasional utilization of mRNA substrates by the process which disperses Alu-like elements. Several β -tubulin pseudogenes in man and probably at least three of the α -tubulin related segments in rat (I.L. and P.A.S. unpublished observations) are processed pseudogenes. Whether tubulin mRNAs are special in their propensity to be duplicated and inserted by this mechanism is not clear. However, if they are typical of mRNAs in general, account for 1% of the total mRNA in a given tissue and are converted into processed pseudogenes at a rate of 1 per 3×10^6 yr, it would appear that the germ line of mammals might be increased by 100 such pseudogenes in this period, not a negligible process when considering alterations in the genetic composition of a species.

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- Cleveland, D. W. *et al.* *Cell* **20**, 95–105 (1980).
- Ponstingl, H., Kraus, E., Little, M. & Kempf, T. *Proc. natn. Acad. Sci. U.S.A.* **78**, 2757–2761 (1981).
- Ginzburg, I., Behar, L., Givol, D. & Littauer, U. Z. *Nucleic Acids Res.* **9**, 2691–2697 (1981).
- Lemischka, I. R., Farmer, S., Racaniello, V. R. & Sharp, P. A. *J. molec. Biol.* **150**, 101–120 (1981).
- Wilde, C. D., Chow, L. T., Wefald, F. C. & Cowan, N. J. *Proc. natn. Acad. Sci. U.S.A.* **79**, 96–100 (1982).
- Cowan, N. J., Wilde, C. D., Chow, L. T. & Wefald, F. C. *Proc. natn. Acad. Sci. U.S.A.* **78**, 4877–4881 (1981).
- Wilde, C. D., Crowther, C. E. & Cowan, N. J. *J. molec. Biol.* **155**, 533–538 (1982).
- Hollis, G. F., Hieter, P. A., McBride, D. W., Swan, D. & Leder, P. *Nature* **296**, 321–325 (1982).
- Van Arsdell, S. W. *Cell* **26**, 11–17 (1981).
- Schmid, C. W. & Jelinek, W. R. *Science* **216**, 1065–1070 (1982).
- Kallayan, L. & Wensink, P. C. *Cell* **24**, 97–106 (1981).
- Sanchez, F., Natzie, J. E., Cleveland, D. W., Kirschner, M. W. & McCarthy, B. J. *Cell* **22**, 845–854 (1980).
- Alexandraki, D. & Ruderman, J. *Molec. Cell. Biol.* **1**, 1125–1137 (1981).
- Mischke, D. & Pardue, M. L. *J. molec. Biol.* **156**, 449–466 (1982).
- Benton, W. D. & Davis, R. W. *Science* **196**, 180–181 (1977).
- Maxam, A. M. & Gilbert, W. *Meth. Enzym.* **65**, 499–580 (1980).
- Sharp, P. A. *Cell* **23**, 643–646 (1981).
- Goldberg, M. thesis, Stanford Univ. (1979).
- Manley, J. L., Fire, A., Cano, A., Sharp, P. A. & Gelfand, M. *Proc. natn. Acad. Sci. U.S.A.* **77**, 3855–3859 (1980).
- Galli, G., Hofstetter, H. & Birnstiel, M. L. *Nature* **294**, 626–631 (1981).
- Barta, A., Richards, R., Baxter, J. D. & Shine, J. *Proc. natn. Acad. Sci. U.S.A.* **78**, 4867–4871 (1981).
- Zakut, R. *et al.* *Nature* **298**, 857–859 (1982).
- Chambon, P. & Breathnach, R. A. *Rev. Biochem.* **50**, 349–383 (1981).
- Proudfoot, N. J. & Browlee, G. G. *Nature* **263**, 211–214 (1976).
- Ben-Ze'ev, Farmer, S. R. & Penman, S. *Cell* **17**, 319–325 (1979).
- Cleveland, D. W., Lopata, M. A., Sherline, P. & Kirschner, M. W. *Cell* **25**, 537–546 (1981).
- Perler, F. *et al.* *Cell* **20**, 555–566 (1980).
- Wilson, A. C., Carlson, S. S. & White, T. J. A. *Rev. Biochem.* **46**, 573–639 (1977).
- Nishioka, Y., Leder, A. & Leder, P. *Proc. natn. Acad. Sci. U.S.A.* **77**, 2806–2809 (1980).
- Wilde, C. D., Crowther, C. E., Cripe, T. P., Gwo-Shu Lee, M. & Cowan, N. J. *Nature* **297**, 83–84 (1982).
- Wilde, C. D., Crowther, C. E. & Cowan, N. J. *Science* (in the press).
- Sutcliffe, J. G., Milner, R. J., Bloom, F. E. & Lerner, R. A. *Proc. natn. Acad. Sci. U.S.A.* **79**, 4942–4946 (1982).
- Elder, J. T., Pan, J., Duncan, C. H. & Weissman, S. H. *Nucleic Acids Res.* **9**, 1171–1189 (1981).
- Grimaldi, G. & Singer, M. *Proc. natn. Acad. Sci. U.S.A.* **79**, 1497–1500 (1982).
- Calos, M. & Miller, J. H. *Cell* **20**, 579–595 (1980).
- Stringer, J. R. *Nature* **296**, 363–364 (1982).
- Steffen, D. & Weinberg, R. A. *Cell* **15**, 1003–1010 (1978).
- Southern, E. J. *molec. Biol.* **98**, 503–517 (1975).
- Vogelstein, B. & Gillespie, D. *Proc. natn. Acad. Sci. U.S.A.* **76**, 615–619 (1979).
- Rigby, P. W. J., Dieckmann, M., Rhodes, C. & Berg, P. *J. molec. Biol.* **113**, 237–251 (1977).
- Davis, R. W., Simon, M. & Davidson, N. *Meth. Enzym.* **21**, 413–428 (1976).
- Li, W. Y., Reddy, R., Henning, P. & Busch, H. *J. biol. Chem.* **257**, 5136–5142 (1982).

LETTERS TO NATURE

Ly α absorption at a high velocity in NGC1275

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The peculiar galaxy NGC1275 = Per A = 3C84 remains a problem in the classification of extragalactic sources despite intensive study in all available wavebands. Included in Seyfert's original list¹, it was described as a Seyfert 2 by Khachikian and Weedman² on the basis of observed emission line profiles. Later Weedman³, however, declined to classify NGC1275 and most recently Véron⁴ has suggested that on the basis of its emission line spectrum, variability and radio and polarimetric properties we should regard NGC1275 as a (rather anomalous) BL Lac object. We present here results of IUE spectrophotometry of NGC1275 which show an absorption feature identified with Ly α at a velocity of 8,200 km s⁻¹. We attribute the origin of this feature to the intervening galaxy responsible for the high velocity emission filaments seen around NGC1275. Profile fitting yields a neutral hydrogen column density in the absorbing medium $N_{\text{H}} \sim 1.8 \times 10^{20}$ atoms cm⁻². Combining this result with available 21-cm absorption data yields a spin temperature $T_s \sim 52$ K for the absorbing gas, typical of that in interstellar H I clouds seen in our Galaxy. The implications of these results are discussed.

In addition to the nuclear emission line spectrum Minkowski⁵ discovered two discrete emission line systems at 5,300 km s⁻¹ and 8,200 km s⁻¹ in the network of filaments surrounding NGC1275. The low velocity (LV) system, at the intrinsic redshift of NGC1275 itself, is roughly symmetrically distributed about the galaxy, while the high velocity (HV) filaments are seen only to the north and west of the nucleus⁶⁻¹⁰. The LV system is thought to arise in condensations in an accretion flow of intracluster gas onto NGC1275⁶, excited either by collisional ionization from shocks generated by colliding clouds or by photoionization by the Seyfert nucleus. Observations of the core of the Perseus cluster with the High Resolution Imager on the Einstein Observatory in an effective range 0.7–3 keV also show evidence for a cooling intracluster flow¹¹, although the relation between the optical and X-ray emission is not fully understood. In contrast to the origin of the LV system, a substantial body of evidence now exists which supports the suggestion that the HV system arises in an infalling late spiral seen along the line of sight to NGC1275 itself⁷. Observations in favour of this hypothesis include: (1) A velocity pattern over the system of HV filaments indicative of a rotating spiral seen almost edge-on⁸. (2) Obscuration of the underlying LV continuum and emission in the HV system are highly spatially correlated. In addition, the Balmer decrement seen in the LV system is much steeper than that seen in the HV system, implying that the latter is intervening⁷. (3) Line strengths in the HV system are typical of those seen in low density, high excitation H II regions excited by stars in the outer regions of late-type galaxies, namely H γ >[N II], [O III]>H β , [N II]~[S II], [O II] λ 3,729>[O II] λ 3,726. In particular, the low

[N II]:[S II] ratio implies a low mass of gas recycled by nucleogenesis in the intervening galaxy⁷. (4) The velocity width of the lines, the H β luminosity and the spatial distribution of excitation level in the HV system are consistent with those seen in spirals⁶. (5) 21-cm radio observations show narrow absorption typical of galactic clouds against the nuclear radio source in NGC1275 at a velocity of 8,200 km s⁻¹ (refs 12–14).

We have studied three spectra of NGC1275 obtained with the SWP camera aboard the IUE satellite¹⁵. The wavelength range covered by these spectra is 1,150–1,950 Å; details of the individual integrations are given in Table 1. The absorption feature identified with Ly α at 8,200 km s⁻¹ was clearly seen in all three spectra, but only SWP 3452 and SWP 4658 were included in the subsequent analysis due to the poor signal-to-noise ratio in SWP 3430. This was due not only to the shorter integration time but also to the intrinsic weakness of the continuum source in NGC1275 at the epoch of this image (see Table 1). Variability in both the continuum and nuclear emission line spectrum in NGC1275 is rapid and complex, and will be discussed elsewhere (S.A.B. *et al.* in preparation).

The spectra were extracted from the geometrically and photometrically corrected (second file) image using the high resolution package developed by Snijders and Adams¹⁶. The wavelength scales in individual spectra differed by <1 Å and after averaging, the resulting absorption profile was modelled using a curve of growth analysis code developed for use in modelling interstellar line profiles¹⁷. Only data points to the red of the central absorption velocity were used as the blue wing of the absorption feature was heavily contaminated by Ly α emission at 5,200 km s⁻¹ associated with the non-thermal Seyfert nucleus. To facilitate comparison with the model profiles the red wing data were reflected about the central absorption velocity (see Fig. 1). Profiles were generated for a range of H I column densities $0.4\text{--}4 \times 10^{20}$ atoms cm⁻², that is well on the damping part of the curve of growth, and were convolved with the IUE instrumental profile at 1,240 Å, which is approximated by a gaussian of FWHM 1,000 km s⁻¹, before comparison with the observed profile.

Figure 1 shows the optimum model profile together with the observed data. The data were normalized to the observed continuum and the abscissa shows velocity in the observer's frame. The feature at $v \sim 11,500$ km s⁻¹ (and reflected at $v \sim 4,900$ km s⁻¹) is due to Si II $\lambda\lambda$ 1,260, 1,264 Å galactic absorption.

The optimal fit was found to correspond to a H I column density of $N_{\text{H I}} = 1.8 (+1.0, -0.8) \times 10^{20}$ atoms cm⁻². De Young *et al.*¹⁴ have derived $N_{\text{H I}} = 3.5 \times 10^{18} T_s$ atoms cm⁻² K, where T_s is the spin temperature of the absorbing material, by study of the 21-cm absorption in the intervening galaxy and combining this with our result we find $T_s = 52 (+28, -24)$ K. If we assume for the absorbing cloud a gas kinetic pressure $NkT = 2 \times 10^3$ k cm⁻³ K as determined for interstellar clouds in the Galaxy¹⁸, then the implied density is $n_{\text{H}} \sim 35$ cm⁻³. These values for both spin temperature and gas density lie within the limits $T_s < 300$ K and $10 < n_{\text{H}} < 100$ cm⁻³ set by Romney¹² for the 21-cm absorbing medium using 21-cm techniques.

Comparing the observed column density with the derived particle density in the cloud, we can estimate a value for the

Table 1 Short wavelength IUE spectra of NGC1275

Image No.	Date year/day	Exposure time (min)	$F_{\lambda}(1,500 \text{ Å})$ ($\times 10^{-14}$ erg cm ⁻² s ⁻¹ Å ⁻¹)
SWP 3430	1978/328	185	0.48 ± 0.07
SWP 3452	1978/329	345	0.81 ± 0.06
SWP 4658	1979/075	250	0.94 ± 0.06

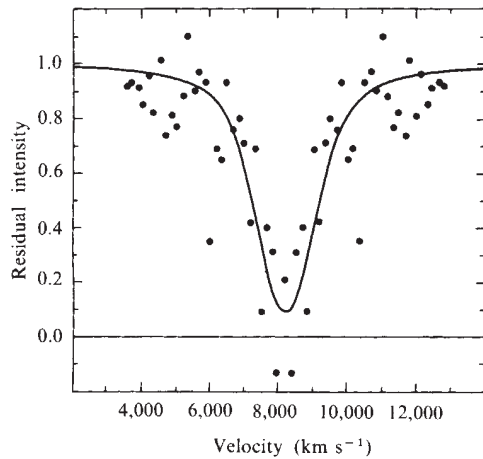


Fig. 1 Absorption due to Ly α at a velocity of 8,200 km s⁻¹ in the UV spectrum of NGC1275. The solid line shows the best fit model profile, corresponding to a neutral hydrogen column density $N(\text{H I}) = 1.8 \times 10^{20}$ atoms cm⁻². Intensity is normalized to the observed continuum and the abscissa shows velocity in the observer's frame. Possible error in centring the absorption feature in velocity space is included in the total error on column density given in the text.

linear dimension of the cloud, $d \sim 2$ pc, consistent with the dimensions of interstellar clouds in our Galaxy. The mean thermal velocity in the cloud is of the order of 1 km s⁻¹, which suggests that the velocity width of 6 km s⁻¹ seen in the 21-cm absorption feature arises as a result of turbulent motion within the cloud. This velocity width is again similar to that seen in clouds in the Galaxy.

The position of the cloud in the intervening galaxy can be estimated from the published map of the high velocity filaments⁸. This suggests that the visible galaxy is of the order of 26 kpc in diameter and is inclined at $i \sim 70^\circ$ to the plane of the sky. The nuclear source in NGC1275 lies at the south-east margin of the HV emission, and our line of sight thus passes through the intervening galaxy at angle of about 20° to the disk at a radial distance of ~ 13 kpc.

The derived density and temperature for the cloud strengthen the conclusion of Kent and Sargent⁶ that the cloud must be distant from the nucleus of NGC1275. Scaling their model by our results shows that the cloud would be destroyed by the nuclear X-ray source in NGC1275 unless it were at least 50 kpc from the source, provided the cloud is not heavily shielded against the X-radiation.

The above results thus confirm the hypothesis that the high velocity system of filaments seen about NGC1275 arises in an intervening galaxy distant from and in line of sight to NGC1275 itself.

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- Seyfert, C. K. *Astrophys. J.* **97**, 28–40 (1943).
- Khachikian, E. Ye. & Weedman, D. W. *Astrophys. J.* **192**, 581–589 (1974).
- Weedman, D. W. *A. Rev. Astr. Astrophys.* **15**, 69–95 (1977).
- Veron, P. *Nature* **272**, 430–431 (1978).
- Minkowski, R. *IAU Symp.* No. 4 107–122 (1957).
- Kent, S. M. & Sargent, W. L. W. *Astrophys. J.* **230**, 667–680 (1979).
- Rubin, V. C., Ford, W. K., Jr., Peterson, C. J. & Oort, J. H. *Astrophys. J.* **211**, 693–696 (1977).
- Rubin, V. C., Ford, W. K., Jr., Peterson, C. J. & Lynds, C. R. *Astrophys. J. Suppl.* **37**, 235–249 (1978).
- Lynds, C. R. *Astrophys. J. Lett.* **159**, L151–L154 (1970).
- Burbidge, E. M. & Burbidge, G. R. *Astrophys. J.* **142**, 1351–1363 (1965).
- Fabian, A. C., Hu, E. M., Cowie, L. L. & Grindlay, J. *Astrophys. J.* **248**, 47–54 (1981).
- Romney, J. D. M. thesis, California Institute of Technology (1978).
- Ekers, R. D., van der Hulst, J. M. & Miley, G. A. *Nature* **262**, 369–370 (1976).
- De Young, D. S., Roberts, M. S. & Saslaw, W. C. *Astrophys. J.* **185**, 809–815, (1973).
- Bogges, A. et al. *Nature* **275**, 372–377 (1978).
- Snijders, M. A. J. & Adams, S. *ESA IUE Newsl.* No. 11, 59–64 (1981).
- Pettini, M. thesis Univ. London, (1978).
- Spitzer, L., Jr. & Jenkins, E. B. *A. Rev. Astr. Astrophys.* **13**, 133–164 (1975).

SAGE—European ozonesonde comparison

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Between February and December 1979, 30 correlative or 'ground-truth' comparisons were conducted of ozone profiles derived from the satellite sensor SAGE (stratospheric aerosol and gas experiment) and those derived from balloon-borne ozonesondes launched in Europe¹. The ozonesonde launches took place at Biscarrosse (44.3° N, 1.2° W), Garmisch-Partenkirchen (47.5° N, 11.0° E), Hohenpeissenberg (47.8° N, 11.0° E), and Lindenberg (52.2° N, 14.1° E). Data comparisons were made when the distance between the SAGE profile and ozonesonde launch location was $< 1,500$ km and the time interval between the measurements ≤ 24 h. This is not an ideal situation but is necessary to accomplish many comparisons without a significant perturbation to the schedules of the ozonesonde launch stations. Even with these large differences in time and space, the comparisons are very good with an average difference between 18 and 28 km of 8.8%. One comparison, however, was within 1 h and a 13 km spatial distance. This was carried out from the 2 April 1979 launch from the ozone station at Garmisch-Partenkirchen. The results are remarkable and together with the general results from the other comparisons are described below.

SAGE uses the technique of limb extinction or solar occultation to infer the vertical distribution of stratospheric ozone. At

Table 1 SAGE-ECC ozone comparisons

Location	Date (1979)	Mean absolute difference (%)
Biscarrosse	7 March	5.5
Biscarrosse	13 March	11.6
Biscarrosse	3 April	12.9
Biscarrosse	3 May	23.0
Biscarrosse	12 June	5.9
Biscarrosse	29 October	4.0
Garmisch	2 April	6.5
Garmisch	10 June	9.2
Hohenpeissenberg	23 February	11.0
Hohenpeissenberg	26 February	6.3
Hohenpeissenberg	28 February	9.5
Hohenpeissenberg	12 March	5.6
Hohenpeissenberg	2 April	6.5
Hohenpeissenberg	4 April	6.9
Hohenpeissenberg	6 April	12.0
Hohenpeissenberg	27 April	9.4
Hohenpeissenberg	30 April	5.7
Hohenpeissenberg	2 May	3.2
Hohenpeissenberg	6 June	14.9
Hohenpeissenberg	11 June	7.5
Hohenpeissenberg	10 October	7.2
Hohenpeissenberg	15 October	7.4
Hohenpeissenberg	17 October	16.3
Hohenpeissenberg	29 October	7.3
Hohenpeissenberg	31 October	6.0
Hohenpeissenberg	24 December	6.5
Hohenpeissenberg	26 December	8.4
Hohenpeissenberg	28 December	14.6
Hohenpeissenberg	31 December	9.4
Lindenberg	28 February	3.4
Average mean absolute difference		8.8%

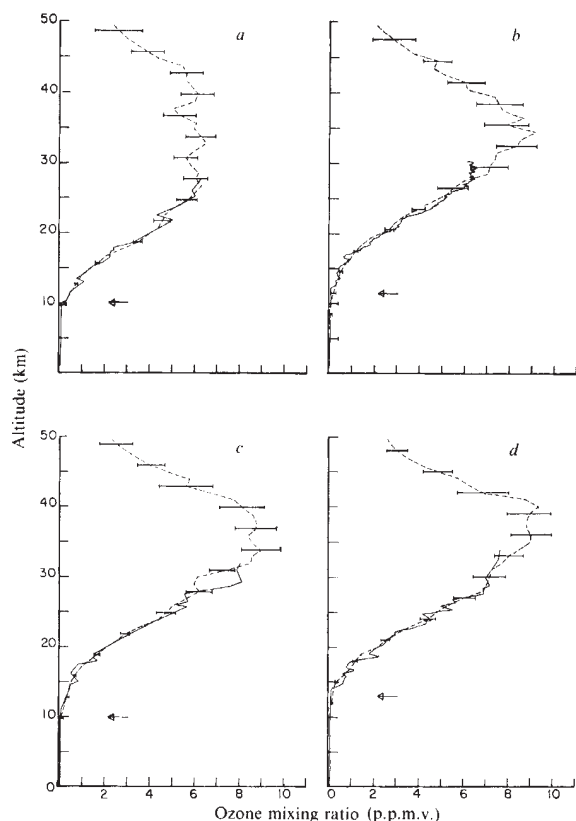


Fig. 1 Ozone comparisons made at four stations in Europe plotted as ozone mixing ratio versus altitude. SAGE data are plotted as the dashed lines and the ozonesonde data as the solid lines. The horizontal lines on the SAGE data indicate the 1σ uncertainties in the SAGE measurements. The horizontal arrow in each comparison plot indicates the altitude of the tropopause. *a*, Lindenbergl, 28 February 1979. The separation (ΔX) at 20 km altitude between soundings is 828 km. The ECC sonde launch time (T_{ecc}) was 1100 GMT while the SAGE measurement time (T_s) was 0643 GMT. *b*, Garmisch-Partenkirchen, 10 June 1979; $\Delta X = 703$ km; $T_{\text{ecc}} = 1716$ GMT; $T_s = 1949$ GMT. *c*, Biscarrosse, 29 October 1979; $\Delta X = 683$ km; $T_{\text{ecc}} = 1331$ GMT; $T_s = 1620$ GMT. *d*, Hohenpeissenberg, 31 October 1979; $\Delta X = 871$ km; $T_{\text{ecc}} = 0706$ GMT; $T_s = 1638$ GMT.

each spacecraft sunrise or sunset, SAGE acquires the Sun and measures its brightness in four spectral bands in the region 0.385–1.0 μm . The radiances are mathematically inverted as shown by Chu and McCormick² to yield profiles of ozone and NO_2 concentration and aerosol extinction with a 1 km vertical resolution on the Earth's horizon. The instrument and experiment are described elsewhere³. The ozonesondes used for these comparisons are variations of the two basic sondes, the Brewer-Mast and Komhyr-ECC sonde⁴. They have been shown to operate well to ~30–35 km with an accuracy of 10% (ref. 5).

Table 1 lists the mean absolute differences between SAGE and the European ozonesondes when ozone mixing ratio (p.p.m.v.) is used as the basis for comparison. The mean absolute difference M was calculated by

$$M = \frac{1}{N} \sum_{i=1}^N \frac{|R_i - r_i|}{(R_i + r_i)/2}$$

where R_i and r_i are the N mixing ratios of SAGE and the European ozonesondes at the SAGE altitudes between 18 and 28 km. Figure 1 shows a composite of profiles typical for each station. The horizontal bars on the SAGE data indicate the expected 1σ uncertainties. The tropopause is indicated by an arrow. The comparisons are quite reasonable, especially considering the large differences in time and space. Figure 2a shows the 2 April 1979 Garmisch comparison where the time and space differences were very small. Two altitude regions show

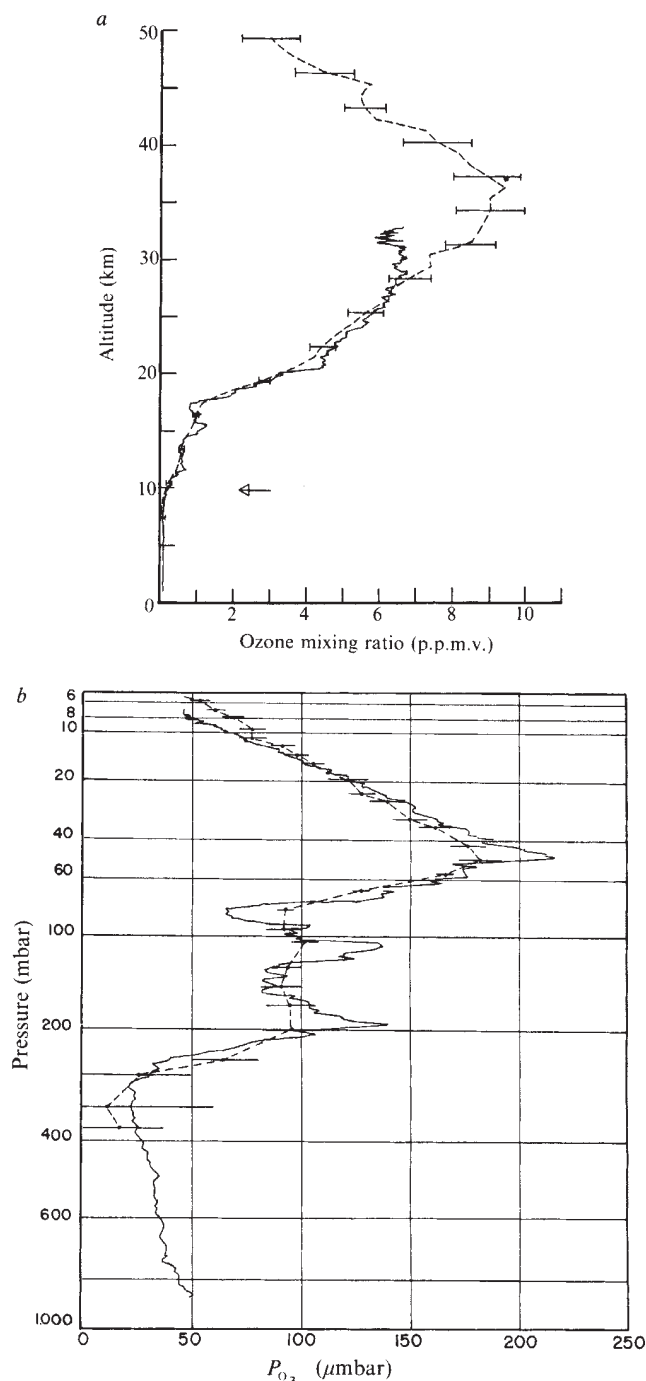


Fig. 2 *a*, Comparison on 2 April 1979, of ozone volume mixing ratio profiles derived from SAGE and an ozonesonde launched from Garmisch-Partenkirchen (47.5° N, 11.0° E). The ECC sonde was launched at 1645 GMT and reached an altitude of 20 km at 1745 GMT. The SAGE measurement was made at 1743 GMT. The horizontal distance between the limb-centroid of the SAGE measurement at 20 km altitude and Garmisch was 13 km. *b*, Comparison as in *a* except ozone is plotted as partial pressure versus pressure altitude.

differences beyond the 1σ expected limits. The fall-off of the ozonesonde data near 30 km is probably due to pump inefficiencies at these altitudes and is characteristic of several ozonesonde soundings. Differences near the tropopause are typical of limb measurement comparisons. Whereas the balloon device samples air as it rises, SAGE integrates its signal over the Earth's limb between the satellite and the Sun. The effective stratospheric pathlength is ~200 km. Over these distances the tropopause and, therefore, the tops of clouds (cirrus and subvisible cirrus) are variable, affecting the measured extinction. In

addition, clouds have occasionally been seen to penetrate the local tropopause. Therefore, we feel that SAGE data should not be compared with *in situ* measurements for the first 1 or 2 km above the indicated tropopause. The mean absolute difference over the 18–28 km range of comparison is only 6.5%. Figure 3 repeats this Garmisch comparison in terms of ozone partial pressure versus pressure altitude to >30 km. The effect of horizontal and vertical integration by SAGE is quite evident, but the agreement is remarkable. For example, the ratio of SAGE to ozonesonde values at 13 out of 17 levels between 12.5 and 28.5 km, lies in the range 0.92–1.06. Allowing for uncertainties in the ozone measurements, differences in vertical resolution, time, and space gradients, this agreement is encouraging.

The SAGE ozone data will be archived beginning in summer 1982 at the United States National Space Science Data Center, Greenbelt, Maryland and the World Ozone Data Center, Toronto, Canada.

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1. *European Ozonesonde Comparisons with the SAGE Satellite System* (NASA, Washington DC, 1982).
2. Chu, W. P. & McCormick, M. P. *Appl. Opt.* **18**, 1404–1413 (1979).
3. McCormick, M. P. *et al. Bull. Am. met. Soc.* **60**, 1038–1046 (1979).
4. *The Stratosphere 1981: Theory and Measurements*, 1–61, (WMO Rep. No. 11, 1981).
5. Attmannspacher, W. & Dutsch, H. U. *Ber. dt. Wetterd.* **120** (1970).

Effects of γ radiation on the leaching kinetics of various nuclear waste-form materials

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When a solid used to immobilize high-level nuclear wastes is evaluated with respect to its long-term durability and effectiveness as a barrier against radio-isotope release, the massive doses of ionizing radiation to which the solid will be exposed during storage have to be taken into account. Unlike particle radiation, which can give rise to considerable damage even in dry conditions¹, γ irradiation seems to enhance leachability significantly only when the solid is in contact with an aqueous medium while being irradiated². Even in these conditions studies of the effect of γ irradiation at dose rates similar to those expected in repository conditions show only a modest radiation-induced enhancement in leach rates. This enhancement, expressed in terms of the ratio $R_{irr} = L_i/L_0$ between the leach rates measured in irradiated and non-irradiated conditions, respectively, is typically of the order of 1–4 in the case of borosilicate glasses containing typical commercial as well as defence waste stream compositions^{3–4}. We show here that the radiation-induced enhancement of leach rates can be ascribed primarily to radiolytic acidification of the aqueous medium and secondarily to radiolytic formation of complexing species. As a result, waste-forms based on major components such as alumina, which are more susceptible to acid dissolution and to attack by complexing agents than silica, show much larger radiation-induced enhancements of their leach rates than silicate glasses.

A series of waste-form materials including PNL 76–68 borosilicate glass³, SRL TDS-131 borosilicate glass⁴, CU PGM high-silica glass² and Synroc-D⁵ were leached inside the cavity

of a ¹³⁷Cs γ source. In each case, a sample with a surface area of $2.5 \times 10^{-4} \text{ m}^2$ was placed in a volume of 0.025 l contained in a 0.030-l polypropylene centrifuge tube closed with a screw cap. The γ dose rate was $8.6 \times 10^4 \text{ rad h}^{-1}$, of the same order of magnitude expected at the surface of a waste-form fully loaded with defense waste⁴. Leachants included de-ionized water and a pH 6 0.05 M phosphate buffer. For each combination of solid and leachant two identical experiments were carried out inside the radiation chamber and two outside the chamber in order to evaluate R_{irr} . The temperature in all cases was $25 \pm 1^\circ \text{C}$. In each case, the leaching period was 3 days, and at the end of this period the leachate was completely removed, analysed and replaced with fresh leachant. This procedure was repeated until five 3-day leaching periods were completed. The experimental error (2σ) in the measurement of leach rates based on Si was up to $\pm 5\%$, and in the case of other elements up to $\pm 20\%$. The results showed that the leach rates usually dropped slightly (by $<30\%$) during the first two intervals and became constant throughout the last three intervals. However, R_{irr} did not show significant dependence on time in any of the experiments.

The results of the leach experiments in the cases of the four waste-form materials in water and in a pH 6 phosphate buffer are shown in Table 1. The first part of Table 1, which summarizes the observed radiation-induced leach rate enhancements in water, shows that the enhancements observed in the cases of the three glasses, which range between 1 and 4, are much smaller than those observed in the case of Synroc-D. This is particularly noticeable when one considers the major structure elements, silica in the case of the glasses and alumina in the case of Synroc-D, which determine the long-term corrosion rate of the bulk material. (In the case of the glasses, the small concentration of alumina in the leachates and the relatively high detection limit (0.05 mg l^{-1} Al as compared with 0.005 mg l^{-1} in the cases of the other elements) did not permit an accurate evaluation of R_{irr}). The moderate enhancements found in the case of the glasses agree with previous findings^{2–4}. In all cases, the final pH of the irradiated leachates was found to be between 4.0 and 4.3. On the other hand, the pH of the unirradiated leachates remained at the level of 5.7–5.8 corresponding to pure water in equilibrium with atmospheric CO_2 .

The second part of Table 1 shows that the enhancements in leach rate become much smaller when the radiation-induced pH decrease is eliminated as a result of buffering the medium at pH 6 using a 0.05 M phosphate buffer. In this case all the enhancements are <2 . This is a very strong and direct indication that the radiation-induced acidification of the aqueous medium is the primary cause of the enhancement of leach rates in γ radiation fields.

Based on this conclusion, the large enhancement in leach rates observed in the case of Synroc-D indicates that this material is much less durable in weakly acid media than the glasses. This is directly confirmed by the findings summarized in the third part of Table 1, which show the ratio R_{ac} between the leach rates obtained in high-dilution non-irradiated leach tests carried on the four materials detailed above in solutions buffered at pH 4 and 6, respectively. (The buffer strength in each case was 0.01 M, and the surface-to-volume ratio was 10 m^{-1} ; to obtain high accuracy, the tests were carried out at 90°C with the data corresponding to the period between the second and the fourth days used in Table 1.) The relative sensitivity of the materials to leaching in a weakly acidified medium agrees very well with their sensitivity to leaching in irradiated pure water as documented in the first part of Table 1.

The different alumina content of the four tested materials is probably the reason for the large differences observed in the sensitivity of their leach rates to pH decrease, whether as the result of irradiation or due to the introduction of acid. The solubility of silica is almost completely independent of the pH in the range 2–7 (ref. 6) and is little affected even in more concentrated acids (except HF, H_3PO_4 and their derivatives)⁷.

Table 1 Radiation effects on leaching and related enhancements

	PNL 76-68 (<0.1% Al ₂ O ₃ , 40.0% SiO ₂)	SRL TDS-131 (3.7% Al ₂ O ₃ , 43.9% SiO ₂)	CU PGM (8.1% Al ₂ O ₃ , 57.3% SiO ₂ , 4.4% P ₂ O ₅)	SYNROC-D (19.0% Al ₂ O ₃ , 6.75% SiO ₂)
R_{irr} (water)				
Si	1.35	3.9	3.5	25
Sr	1.0	1.5		15
Ca	2.3	1.7		36
Na	2.8	1.2	4.2	9
Al				113
R_{irr} (pH 6 buffer)				
Si	1.15	1.2	1.45	1.4
Sr	1.5	1.0		1.0
Ca	0.7	1.2		1.1
Al				1.75
R_{ac} (pH 4/pH 6)				
Si	2.2	11.0	6.5	2553
Sr	12.4	3.9	30	235
Ca	5.6	6.7	7.7	194
Al				66
R_{oxal} (0.05 M/0.01 M)				
Si	2.0	6.9	0.7	3.4
Ca	2.0	7.5	0.7	0.9
Al		6.6	0.9	3.5
Ti	2.3	6.9		11

On the other hand, the solubility of alumina rises very sharply when the pH decreases (by factors of 3, 26 and 1,800 at pH levels of 6, 5 and 4, respectively, relative to the solubility at pH 7)⁸. The sensitivity of silicate solids and glasses⁷ to acid dissolution grows rapidly and progressively⁹ with the introduction of alumina, and aluminosilicate minerals display a pH dependence almost as sharp as the one observed with pure gibbsite¹⁰.

In the present case, the rise of R_{irr} and R_{ac} with alumina content is roughly exponential, showing a similarity to the data in ref. 9. Focusing again on data based on the major structure elements, it can be seen that in the case of PNL glass, which contains only traces of alumina, the dissolution rate (based on Si) increases only by a factor of 1.35 when leaching is carried out in a radiation field. For SRL and CU glasses, which have an Al₂O₃ content of 4–8%, the corresponding R_{irr} ratios are between 3 and 4, and in the case of Synroc-D (19% alumina) R_{irr} based on Si is 25 but R_{irr} based on Al, the major matrix element in the latter case, is as high as 113. A minor discrepancy is shown by the finding that CU glass is not more sensitive to the presence of acid (as indicated both by R_{irr} and by R_{ac}) than SRL glass despite the higher alumina content of the former. This can be attributed to the presence of a relatively high concentration of P₂O₅ in the case of CU glass alone, as AlPO₄ is much less soluble than Al₂O₃ in moderately acidic media¹¹. In addition, the high silica content of CU glass is likely to strengthen the matrix against acid attack.

The formation of acidic species on irradiation of aerated water has been observed earlier^{3,4}, and the pH decrease appears to be larger the higher the radiation dose. Earlier work showed that both nitric acid^{3,4} and hydrogen peroxide³ appear in the irradiated medium. Present findings show the presence of HNO₃, HCOOH, (COOH)₂ and H₂O₂ at concentrations of 78, 46, 30 and 16 $\mu\text{mol l}^{-1}$, respectively, at the end of a 3-day irradiation period in the conditions detailed above. In addition, small amounts of higher organic acids have been detected. The formation of organic acids in aqueous carbon dioxide solutions previously reported^{12,13} is likely to result in the present case from reduction of dissolved atmospheric CO₂ by hydrated electrons, which are highly reactive towards CO₂ (ref. 14). The mechanism of HNO₃ formation from dissolved atmospheric N₂ and, possibly, O₂ is less clear, but preliminary doping experiments with alcohols and the observation that hydrated electrons are not reactive towards N₂ (ref. 15) indicate that hydroxyl radicals may be involved.

In any case, the formation of nitric acid may not be very significant in repository conditions in the event that the replenishment of atmospheric gases, after the chamber which contains the waste packages has been sealed, proves to be slow. On the other hand, the fact that at least one half of the total acid content in irradiated water is due to the formation of carboxylic acids from dissolved CO₂ implies that enhanced leaching due to radiolysis may prove to be a persistent effect due to the presence of significant levels of CO₂ and carbonate species in ground-water in general¹⁶. The extent of carboxylic acid formation in a particular ground-water composition is likely to depend on the concentration of carbonate species as well as on the pH.

Furthermore, the radiation-induced formation of oxalic acid and of other organic acids, as well as the possible formation of aldehyde and glycol¹³, raises the possibility that the radiation-induced increase in leach rate is due in part to specific effects of complexing species such as oxalate. It is well-known that glasses undergo accelerated attack in the presence of complexing agents¹⁷.

As the data in the second part of Table 1 demonstrate, leach rates are enhanced by factors ranging up to almost 2 even when the pH decrease is suppressed by means of a buffer. This residual enhancement probably reflects the effect of the formation of oxalate and other complexing species. The fourth part of Table 1 shows the results of measurements of R_{oxal} , the enhancement of leach rates in the presence of oxalate. The measurements were carried out using the same procedure as that used in the non-irradiated leach experiments used in the determination of R_{irr} (25 °C, surface-to-volume ratio = 10 m⁻¹, five 3-day leach periods). R_{oxal} represents the ratio between the leach rates obtained in a 0.05 M pH 5 Na oxalate-oxalic acid buffer and in the same buffer diluted by a factor of 5 with de-ionized water, respectively. The results show significant enhancements in leach rates in all cases, except CU glass, when the samples are exposed to the more concentrated oxalate buffer. The lack of effect in the case of CU glass may be related to the high concentration of silica, which forms a dense network insensitive to complexants. On the other hand, SRL glass and Synroc-D, which exhibit high values of R_{oxal} , have high concentrations of complexable species such as Al and Ti. The build-up of radiation-produced complexing species, in particular oxalate, in ground-water is probably most significant with respect to the leaching of actinides, lanthanides and transition metal ions which are strongly complexed by oxalate.

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1. Dran, J. C., Maurette, M., Petit, J. C. & Vassent, B. in *Scientific Basis for Nuclear Waste Management* Vol. 3 (ed. Moore, J. G.) 449-456 (Plenum, New York, 1981).
2. Barkatt, A., Simmons, J. H. & Macedo, P. B. *Phys. Chem. Glasses* **22**, 73-85 (1981).
3. McVay, G. L. & Pederson, L. R. *J. Am. ceram. Soc.* **64**, 154-158 (1981).
4. Walker, D. D., Dukes, M. D., Plodinec, M. J. & Bibler, N. E. *Symp. on Chemical Consideration for Important Radioactive Waste Species*, Atlanta (1981).
5. Newkirk, H. W. et al. *J. Am. ceram. Soc.* (in the press).
6. Alexander, G. B., Heston, W. M. & Iler, R. K. *J. phys. Chem.* **58**, 453-455 (1954).
7. Adams, P. B. in *Ultrapurify* (eds Zief, M. & Speights, R. M.) Ch. 14 (Dekker, New York, 1972).
8. Dezelic, N., Bilinski, H. & Wolf, R. H. *J. inorg. nucl. Chem.* **33**, 791-798 (1971).
9. Ohta, H. & Suzuki, Y. *Bull. Am. ceram. Soc.* **57**, 602-604 (1978).
10. Tole, M. P. thesis, Pennsylvania State Univ. (1982).
11. Ghani, M. O. & Aleem, S. A. *Ind. J. agr. Sci.* **13**, 142-147 (1943).
12. Garrison, W. M., Morrison, D. C., Hamilton, J. G., Benson, A. A. & Calvin, M. *Science* **114**, 416-418 (1951).
13. Getoff, N. *Int. J. Appl. Rad. Isotopes* **13**, 205-213 (1962).
14. Gordon, S., Hart, E. J., Matheson, M. S., Rabani, J. & Thomas, J. K. *Disc. Faraday Soc.* **36**, 193-205 (1963).
15. Shaede, E. A., Edwards, B. F. P. & Walker, D. D. *J. phys. Chem.* **74**, 3217-3220 (1970).
16. White, D. E., Hem, J. D. & Waring, G. A. in *Data of Geochemistry* (ed. Fleischer, M.) (U.S. Government Printing Office, Washington DC, 1963).
17. Ernsberger, F. M. *J. Am. ceram. Soc.* **42**, 373-375 (1959).

The hydrophobic interaction is long range, decaying exponentially with distance

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The attractive interaction between organic nonpolar molecules, such as hydrocarbons, in water is unusually strong. This 'hydrophobic interaction'¹ is responsible for the very low solubility of hydrophobic molecules in water, and has a central role in micelle formation, biological membrane structure, and in determining the conformations of proteins^{2,3}. It was once believed that because the interaction is so strong there is a 'hydrophobic bond' associated with it⁴; but it is now recognized that the interaction involves the configurational rearrangement of water molecules as two hydrophobic species come together⁵⁻⁹ and is therefore of longer range than a typical covalent bond. However, there has been no experimental information available concerning the distance dependence and effective range of this interaction. From measurements of the total force as a function of distance between two hydrophobic surfaces immersed in aqueous electrolyte solutions we have determined accurately the attractive component due to the hydrophobic interaction and found that the hydrophobic interaction has the same range as, but is about an order of magnitude stronger than, the van der Waals-dispersion force; and that in the range 0-10 nm it decays exponentially with distance with a decay length of ~1 nm. The results can be roughly extrapolated to molecular interactions and show that the interaction free energy of two hydrophobic solute molecules of radius R (nm) in water at 21 °C is approximately given by $\Delta G_H = -40R$ kJ mol⁻¹, which is in agreement with previous estimates. However, the hydrophobic interaction is not due to a 'hydrophobic bond', and its long-range nature has obvious implications for the mechanism and rates of folding as well as the equilibrium conformations of proteins and other macromolecules.

We have measured the forces between two hydrophobic surfaces in aqueous solutions in the distance range $D = 0$ (contact) to $D > 50$ nm. The experimental techniques used were similar to those used previously for measuring the forces between mica surfaces in liquids¹⁰⁻¹⁴, which allow for the force F between two crossed cylinders of mica of radius R to be measured with a distance resolution of about 0.2 nm. In the present experiments the mica surfaces were rendered hydrophobic by the adsorption of a monolayer of the cationic surfactant hexadecyltrimethylammonium bromide, $\text{CH}_3-(\text{CH}_2)_{15}-\text{N}(\text{CH}_3)_3^+\text{Br}^-$ or CTAB, from solution. At concentrations just below the CMC (10^{-3} M) the positive CTA^+ group adsorbs onto the negatively charged mica surface as a compact monolayer exposing a hydrophobic surface composed of CH_3 and CH_2 groups¹². In this range of concentrations the net surface charge on the mica⁻-CTA⁺ surface goes from being negative to positive, passing through its point of zero charge (PZC). Thus in addition to any attractive force between two such monolayer-covered surfaces there is a repulsive electric 'double-layer' force arising from the incomplete charge neutralization at the mica⁻-CTA⁺ interface. We have measured how these forces vary with distance for a range of CTAB concentrations in both distilled water and in NaCl and KBr solutions up to 0.1 M and at various pH values in the range 5.4-10.4.

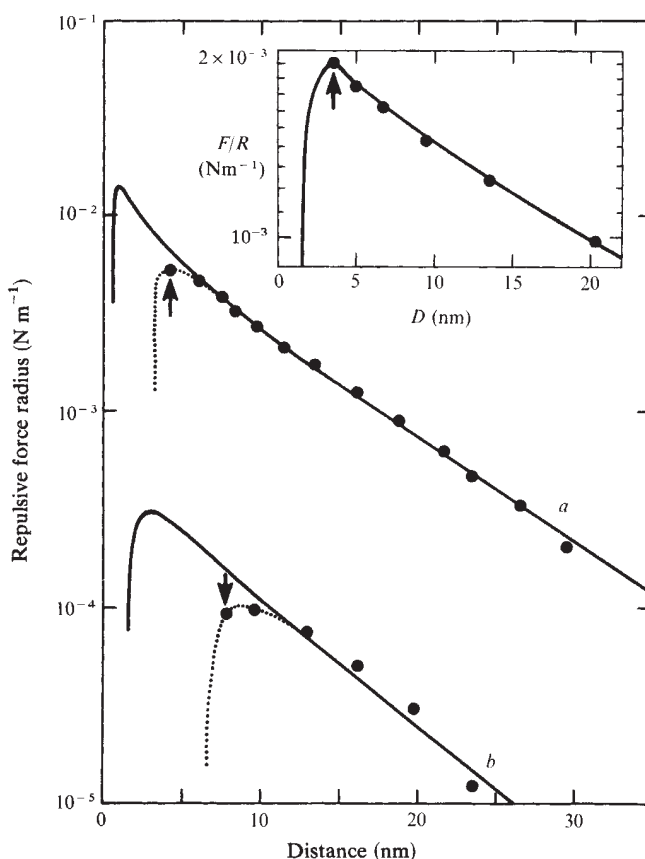


Fig. 1 Measured repulsive force F between two cylindrically curved hydrophobic surfaces (of radii R) as a function of distance D in $\sim 10^{-3}$ M NaCl and KBr solutions at 21 °C (plotted as normalized force F/R). The effective surface charge density corresponding to the observed double-layer forces are $1e$ per 5 nm^2 (a), and $1e$ per 95 nm^2 (b). The solid lines are the theoretical force laws expected from the classical DLVO theory^{15,16}. Dotted lines, experiment force laws. Inset: measured force law between uncoated mica surfaces where excellent agreement with the DLVO theory is obtained. Errors in distance measurements are negligible (± 0.2 nm); errors in the force are roughly equal to the size of the experimental points, except at smaller distances where the errors are smaller.

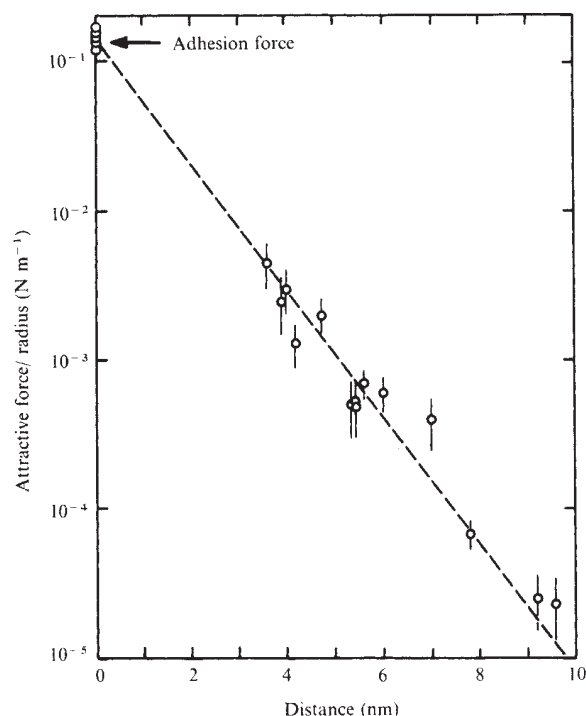


Fig. 2 Attractive force law F_H as a function of distance D as deduced from a wide range of force curves such as those shown in Fig. 1 (and for different values of R between 0.75 and 2.0 cm). Experimental points are obtained from the differences between the measured and theoretical forces in the regions of the observed maxima (arrows in Fig. 1). The hydrophobic interaction decays exponentially out to a separation of about 10 nm, and is given by $F_H/R = 0.14 e^{-D/1.0} \text{ N m}^{-1}$.

Figure 1 shows the measured forces in two instances: high surface charge, and very low surface charge (near the PZC). In each case (and in many others, not shown) the attractive component of the interaction greatly exceeds that expected from the normal van der Waals-dispersion contribution to the total van der Waals + double layer (DLVO) interaction^{15,16}, which is shown as the solid curves in Fig. 1. This is in marked contrast to similar measurements of forces between uncoated mica surfaces in dilute electrolyte solutions^{11,14} where the agreement with the DLVO theory is excellent (Fig. 1 inset).

By subtracting the measured forces from the theoretical curves one can deduce the additional attractive force between the hydrophobic surfaces at different values of D down to the 'force barriers' (arrows in Fig. 1), below which the net force becomes rapidly attractive and the two surfaces jump into monolayer contact at $D = 0$.

The additional attractive (hydrophobic) force is plotted as F_H/R against D in Fig. 2, where we have also included the adhesion or 'pull-off' force at $D = 0$, given by the force needed to separate the two surfaces from adhesive contact. It is apparent that over the distance range 0–10 nm the hydrophobic force-law is well described by an exponential function given by

$$F_H/R = C e^{-D/D_0}$$

where $C = 0.14 \pm 0.02 \text{ N m}^{-1}$, and decay length $D_0 = 1.0 \pm 0.1 \text{ nm}$.

We found that the strength of this interaction is not sensitive to the type and concentration of the electrolyte present, nor to the pH. The adhesion force F_H/R at $D = 0$ did, however, increase slightly when the repulsive double-layer forces were weaker, as might be expected. Experiments were also performed with the surfactant concentration progressively diluted to 1/100 of the CMC with no resulting change in the attractive

force-law, thus ruling out the possibility that the presence of surfactants is responsible for the extra attraction (through some bridging mechanism involving premicellar aggregates, for example).

The measured attractive force-law is about an order of magnitude larger than the maximum possible van der Waals-dispersion force; and since it decays exponentially with distance rather than as a power law it cannot be attributed to a 'modified' dispersion interaction. It is most likely that this force is the 'hydrophobic interaction'. Although we cannot discuss here all the implications arising from this work or correlate our results with previous experimental and theoretical studies the following three points are notable:

(1) The hydrophobic interaction acts over long-range and cannot be considered as arising from any bond-like association. For two surfaces 50% of the total interaction free energy occurs at distances beyond about two water molecule diameters. The exponential dependence of the interaction has been predicted in recent theories of solvation forces^{7,8}.

(2) The measured force law given above corresponds to a pair interaction free energy of

$$\begin{aligned} \Delta G_H &= -CRD_0 e^{-D/D_0} \\ &\approx -84R e^{-D/D_0} \text{ kJ mol}^{-1} \quad (R \text{ in nm}) \end{aligned}$$

for two crossed cylinders or for a sphere of radius R near a flat surface, while more generally the value of R above should be replaced by $R_1 R_2 / (R_1 + R_2)$ for two interacting spheres of radii R_1 and R_2 . Although there are obvious objections to extrapolating this expression down to molecular radii, it does give surprisingly good values when used to estimate the hydrophobic interaction of small solute molecules in water. Thus for two dissolved methane molecules (hard sphere radius $R = 0.18 \text{ nm}$)^{17–19}, two benzene molecules ($R = 0.25 \text{ nm}$)^{17,18}, and two cyclohexane molecules ($R = 0.28 \text{ nm}$)^{17,18}, the values obtained for the free energy of dimerization ($\Delta G_H \approx -42R \text{ kJ mol}^{-1}$ at $D = 0$) in each case are $\Delta G_H \approx 7.6, 10.5$ and 11.8 kJ mol^{-1} , respectively, which compare surprisingly well with the various theoretical and experimentally determined values of 7.5–8.8 (refs 5, 6), 8.5–9.6 (refs 9, 20) and 11.3 (ref. 20) kJ mol^{-1} .

(3) The long-range attractive forces measured between surfactant¹² and lipid bilayers^{21,22}, where both hydrophilic and hydrophobic groups are present at these interfaces, appear to be well described by the van der Waals-dispersion force, suggesting that the hydrophobic interaction is neutralized when the local structure of water molecules is dominated by their interaction with nearby hydrophilic groups.

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1. Franks, F. *Water: A Comprehensive Treatise* Vol. 4 (ed. Franks, F.) Ch. 1 (Plenum, New York, 1973).
2. Kauzmann, W. *Adv. Protein Chem.* **14**, 1–63 (1959).
3. Tanford, C. *The Hydrophobic Effect* 2nd edn (Wiley, New York, 1980).
4. Nemethy, G. & Scheraga, H. A. *J. phys. Chem.* **66**, 1773–1789 (1962).
5. Ben-Naim, A., Wilf, J. & Yaacobi, M. *J. phys. Chem.* **77**, 95–102 (1973).
6. Dashesky, V. G. & Sarkisov, G. N. *Molec. Phys.* **27**, 1271–1290 (1974).
7. Marcelja, S., Mitchell, D. J., Ninham, B. W. & Sculley, M. J. *JCS Faraday Trans. II* **73**, 630–648 (1977).
8. Chan, D. Y. C., Mitchell, D. J., Ninham, B. W. & Pailthorpe, B. A. *Molec. Phys.* **35**, 1669–1679 (1978).
9. Rossky, P. J. & Friedman, H. L. *J. phys. Chem.* **84**, 587–589 (1980).
10. Israelachvili, J. N. & Adams, G. E. *Nature* **262**, 774–776 (1976); *JCS Faraday Trans. I* **74**, 975–1001 (1978).
11. Pashley, R. M. *J. Colloid Interface Sci.* **80**, 153–162 (1981).
12. Pashley, R. M. & Israelachvili, J. N. *Colloids & Surfaces* **2**, 169–187 (1981).
13. Horn, R. G. & Israelachvili, J. N. *J. chem. Phys.* **75**, 1400–1411 (1981).
14. Israelachvili, J. N. *Phil. Mag.* **A43**, 753–770 (1981).
15. Verwey, E. J. W. & Overbeek, J. Th. G. *Theory of the Stability of Lyophobic Colloids* (Elsevier, New York, 1947).
16. Chan, D. Y. C., Pashley, R. M. & White, L. R. *J. Colloid Interface Sci.* **77**, 283–285 (1980).
17. Evans, D. F., Tominaga, T. & Davis, H. T. *J. chem. Phys.* **74**, 1298–1305 (1981).
18. Dymond, J. H. *J. phys. Chem.* **85**, 3291–3294 (1981).
19. Harris, K. R. & Trautenberg, N. *J. Physica* **104A**, 262–280 (1980).
20. Tucker, E. E., Lane, E. H. & Christian, S. D. *J. Solut. Chem.* **10**, 1–20 (1981).
21. Parsegian, V. A., Fuller, N. & Rand, R. P. *Proc. natn. Acad. Sci. U.S.A.* **76**, 2750–2754 (1979).
22. Ohshima, H., Inoko, Y. & Mitsui, T. *J. Colloid Interface Sci.* **86**, 57–72 (1982).

Oscillatory spreading explanation of anomalously old uplifted crust near oceanic transforms

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Rocks significantly older than their theoretically predicted age have been recovered in the vicinity of some Atlantic Transform Zones. Rocks of Palaeocene (55–58 Myr) and possibly even of Cretaceous age are found near the Vema Transform, on tectonically uplifted crust which should not be older than 30 Myr. We present here a model which might explain the presence of uplifted, anomalously old crust near the Vema Transform Zone (TZ). The proposed model involves axial rift propagation and reorientation and migration of the transform as a result of a change in the regional stress field. During transform migration a crustal wedge, formerly part of the African plate, is transferred to the South American plate. The ensuing oscillatory spreading can explain the anomalous age of the uplifted crustal block. Compression resulting from rift propagation and transform migration provides a mechanism for crustal uplift.

Two fundamental tenets of the theory of plate tectonics are: (1) the age of the oceanic crust increases with distance from the axis of crustal accretion (the axis of mid-oceanic ridges) where the age is close to zero; and (2) the depth below sea level of the oceanic basement increases proportionally to the square root of the age of the crust, due to cooling and thermal contraction of the oceanic lithosphere during its motion away from the axis of accretion. These two tenets are supported by a wealth of observations obtained from the three major ocean basins. However, exceptions exist. In particular, crustal blocks which have been subjected to intense vertical tectonic movements and reach anomalously shallow depths have been observed in the vicinity of large offset ridge–ridge transform zones in the Atlantic and Indian Oceans^{1–3}. Moreover, rocks significantly older than their theoretically predicted age have been reported from the vicinity of some Atlantic transform zones, namely the Atlantis TZ at 29° N (ref. 4), the St Paul TZ to 1° N (ref. 5) and the Vema TZ at 11° N (refs 6–8). No

satisfactory explanation has been presented to date, which would reconcile seafloor spreading with the occurrence of old material close to the axis of lithospheric accretion.

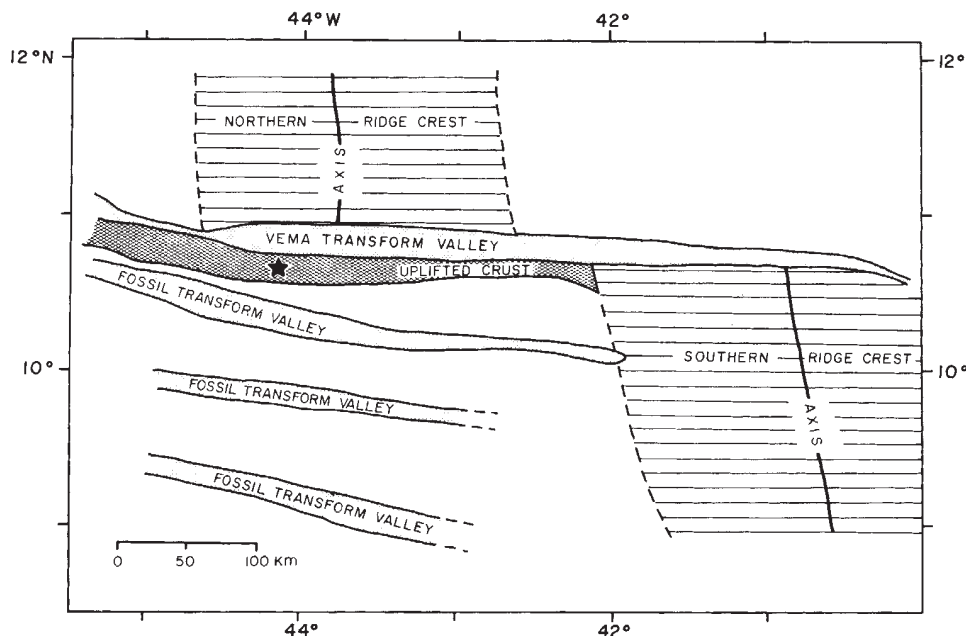
The Vema TZ offsets the axis of crustal accretion by ~300 km (Fig. 1). The transform valley is filled with nearly 1 km of sediment; the recent strike-slip transform motion can be traced in the sediment close to the axis of the transform valley⁹. To the south of the transform valley and parallel to it, lies a narrow ridge (transverse ridge) whose summit is shallower than 600 m below sea level^{10,11}. The ridge constitutes a major topographic anomaly relative to the predicted depth–age relationship (Fig. 2), and is probably a tectonically uplifted sliver of crust and upper mantle^{11,12}.

The summit of the uplifted ridge is capped by shallow-water reef limestones, some of which have undergone sub-aerial diagenesis. Palaeofacies studies and age determinations of these limestones indicate that the summit of the uplifted block was above sea level from late Miocene up to middle Pliocene time (from ~10 to 3 Myr BP). It subsequently subsided at an average rate of 0.3 mm yr⁻¹, a rate one order of magnitude faster than predicted by thermal contraction of the plate⁸.

Although equatorial magnetic anomalies are not very well developed, they are good enough to estimate spreading rates. Values ranging from 1.1 to 1.4 cm yr⁻¹ have been determined from profiles located immediately north of the transform^{11,13,14}. Assuming this spreading rate is correct, and that the uplifted crust on the southern side of the transform valley was originally created at the southeastern axial segment of the Mid-Atlantic Ridge, an age of 30–35 Myr can be estimated for the area of maximum uplift on the transverse ridge (Fig. 3). However, deep-sea limestones were recovered from the uplifted block (in addition to the shallow water samples), which have been dated as early Palaeocene (~55–58 Myr BP) on the basis of their fossil planktonic foraminiferal fauna⁸. This anomalously old age is consistent with ages determined from other limestone samples bearing pre-Tertiary fossil crab coprolites of the genus *Parafavosites*^{6,7}.

It is possible that the anomalously old crust is related to reorientation and migration of the transform in the past. A set of sediment-filled valleys south of, and striking 10°–15° oblique to the Vema Transform (Fig. 1) were identified and interpreted as traces of former transforms by Van Andel *et al.*^{11,13}. These fossil transform traces are present only in crust older than ~10 Myr. The different orientation of the former transforms relative to the presently active Vema TZ suggests that a reorientation by 10°–15° of the direction of spreading occurred

Fig. 1 Primary morphotectonic features at the Vema TZ in the Atlantic, after refs 11, 13. ★, Site of maximum uplift (<600 m below sea level) on transverse ridge.



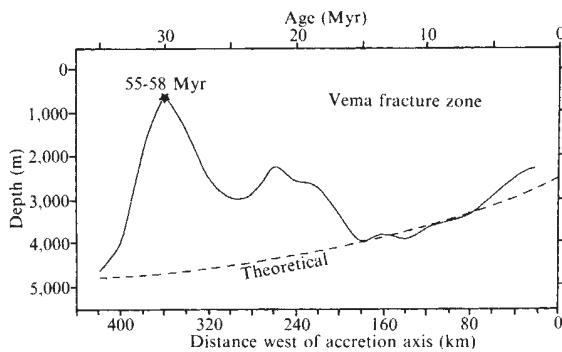


Fig. 2 Topography versus age on a profile immediately south of the Vema Transform valley, compared with the theoretical curve²³ predicted for thermal contraction of the lithosphere. A spreading rate of 1.2 cm yr^{-1} is assumed.

in this area ~ 10 Myr BP (ref. 13). This reorientation is also suggested by both a change in the trend of the western extension of the Vema fracture zone, and by a corresponding change in strike of the 10-Myr old magnetic anomalies¹³.

Based on this information, we propose the following sequence of events to explain the existence of anomalously old and tectonically uplifted crustal blocks along the transform (Fig. 3).

(a) Before ~ 10 Myr ago a ridge-ridge transform zone was active to the south of the present Vema TZ. The strike of the former transform was 10° – 15° NW of the present transform strike.

(b) A change in spreading direction of 10° – 15° occurred, probably related to a reorientation of the lithospheric regional stress field. In response to the changing stress field, the south-east segment of the accretion axis gradually propagated northward. This northward propagation of the axis of spreading might have taken place in a manner similar to that suggested for the Juan de Fuca ridge, the Galapagos rift and the East Pacific Rise^{15–18}. According to Fujita and Sleep¹⁹ and Crane and Gallo²⁰ the magnitude and direction of intraplate stress acting on the ridge-transform system may be the primary cause for the readjustment of a transform and the reorientation of the zone of extension.

(c) A new ridge-transform-ridge geometry, similar to that of the present Vema TZ became established ~ 10 Myr BP.

(d) Spreading in an east-west direction has occurred for ~ 10 Myr. The fossil transform valley can be traced south of the presently active Vema TZ.

We stress that the geometry outlined in the evolutionary scheme of Fig. 3 can be modified without changing the substance of the model. For instance, we have assumed that the change in orientation of the axis of spreading and of the transform pivots around a fixed point at the southern tip of western axis of spreading. This assumption may not be strictly true.

One consequence of the model illustrated by Fig. 3 is that a portion of crust close to the northern side of the former transform boundary moved east-south-east before the reorientation of spreading direction (Fig. 3a). After the migration of the transform to the north, this wedge of crust was transferred from the African to the South American plate and thus began to move to the west (Fig. 3c, d). We refer to this reversal of spreading direction as 'oscillatory spreading', and we suggest that this might explain the presence of anomalously old crust on the southern side of the Vema TZ.

Considering the location and age of the anomalously old rocks, assuming an average spreading rate of 1 cm yr^{-1} or more, and assuming that the present spreading direction became established roughly 10 Myr ago, it follows that the 55–58-Myr old crust cannot be explained simply by one cycle of instantaneous transform reorientation and migration, as depicted in Fig. 3. The simple model of Fig. 3 can explain the 55–58-Myr

old crust if there was a transitional period of reorientation and migration of the transform lasting several million years, during which the wedge of crust between the old and the new transform would remain more or less stationary. Alternatively, the anomalous 55–58-Myr old crust might have been involved in more than one stage of migration of the transform by rift propagation. The presence of crust as old as Cretaceous, if confirmed, would support the idea that several transform migration steps have occurred. The existence of at least three fossil transform valleys to the south of the presently active Vema TZ (Fig. 1) suggests indeed that the northward transform migration by rift propagation occurred in more than one step.

The observation that the presently active transform valley is filled by sediments younger than a few million years^{11,21} supports a young age for the Vema Transform Valley and is consistent with the suggested model.

Transform reorientation and migration might be responsible also for the vertical tectonic motions which gave rise to the uplifted crust at the Vema TZ. Vertical tectonic movements of crustal blocks can occur in the vicinity of a transform zone due to several distinct processes^{12,18,22}. One process would involve small changes in spreading direction, which result either in

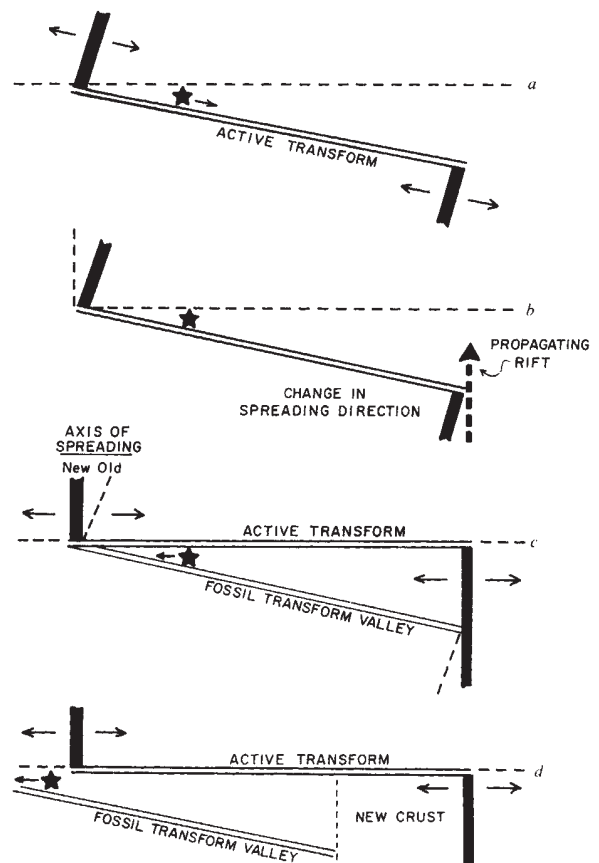


Fig. 3 Proposed model on transform migration and oscillatory spreading. *a*, Former ridge-transform-ridge geometry. Crust at site $\star$ is part of African plate and moves east-southeast. *b*, A change of 10° – 15° in spreading direction occurs. Southern ridge axis starts propagating northward. *c*, Situation 10 Myr ago. Migration and reorientation of transform has taken place. Site $\star$, now part of South American plate, reverses its motion and starts to move to the west giving rise to oscillatory spreading. *d*, Present situation, site $\star$ continues to move westward. Fossil transform valley present south of the present transform. To explain the ages obtained from the summit of the uplifted crustal sliver, more than one cycle of transform migration is required. Alternatively a long (several million years) period of transition between stages *a* and *c* is necessary.

compression or extension along the fracture zone. Within our proposed model of rift propagation and transform reorientation and migration, compressional regimes are likely to occur in portions of the crustal wedge situated between the old and the new transform. The wedge should be subjected to maximum compression during the time of transition from the old to the new ridge-transform-ridge geometry (stage *b* in Fig. 3). Crustal uplift might have occurred in response to the compressional regime. Note that the summit of the uplifted block stayed above sea level from at least mid-Miocene (~10 Myr BP) to mid-Pliocene (3 Myr BP) time⁸. These ages are consistent with a model which predicts the subsidence of the block after the present ridge-transform-ridge configuration became established, ~10 Myr BP. Uplift took place before 10 Myr ago.

A possibility to be considered is that a compressional regime develops in front of a propagating rift. In cases where the rift

is unable to advance, compressional stresses at its tip may induce vertical movements of the crust. It is possible that during the reorientation of the Vema ridge-transform system, while the south-east axis of accretion was able to propagate northwards, the north-west axis did not propagate to the south but created compression and crustal uplift on its front across the transform. In fact, we note that the uplifted crustal block is located roughly across the transform from the north-west axis of spreading.

A more precise assessment of the processes which led to crustal uplift and presence of anomalously old crust at the Vema TZ may allow us to use the proposed model to explain transform related anomalous ages and vertical tectonics in other areas.

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- Bonatti, E., Chermak, A. & Honnorez, J. in *2nd Ewing Symp.* Vol. 239-248 (American Geophysical Union, Washington DC, 1979).
- Bonatti, E. & Chermak, A. *Tectonophysics* **72**, 165-180 (1981).
- Bonatti, E. & Hamlyn, P. R. *Science* **201**, 249-251 (1978).
- Ozima, M. *et al.* *Tectonophysics* **31**, 59-71 (1976).
- Melson, W. G., Hart, G. R. & Thompson, G. *Geol. Soc. Am. Mem.* **132**, 241-272 (1972).
- Bonatti, E. & Honnorez, J. *Science* **174**, 1329-1331 (1971).
- Honnorez, J. *et al.* *Earth planet. Sci. Lett.* **26**, 8-12 (1975).
- Bonatti, E., Sartori, R. & Boersma, A. *Tectonophysics* (in the press).
- Ettreim, S. & Ewing, J. *Geology* **3**, 555-558 (1975).
- Van Andel, T. H., Corliss, J. B. & Bowen, V. T. *J. mar. Res.* **25**, 343-351 (1967).
- Van Andel, T. H., Von Herzen, R. D. & Phillips, J. D. *Mar. geophys. Res.* **1**, 261-283 (1971).
- Bonatti, E. *Earth planet. Sci. Lett.* **37**, 369-379 (1978).
- Van Andel, T. H., Phillips, J. D. & Von Herzen, R. P. *Earth planet. Sci. Lett.* **5**, 296-300 (1969).
- Bonatti, E. & Honnorez, J. *J. geophys. Res.* **81**, 4104-4116 (1976).
- Hey, R. N. *Earth planet. Sci. Lett.* **37**, 321-332 (1977).
- Crane, K. J. *geophys. Res.* **84**, 6011-6018 (1979).
- Hey, R. N., Duennebie, R. K. & Morgan, W. J. *J. geophys. Res.* **85**, 3647-3658 (1980).
- Crane, K. *Mar. Geol.* **21**, 25-46 (1976).
- Fujita, K. & Sleep, M. H. *Tectonophysics* **50**, 207-221 (1978).
- Crane, K. & Gallo, D. J. *geophys. Res.* (in the press).
- Bader, R. G. *et al.* *Init. Rep. DSDP Leg 4*, 77-92 (1970).
- Sleep, N. H. & Biehler, S. J. *geophys. Res.* **75**, 2748-2752 (1971).
- Slater, J. G., Anderson, R. M. & Bell, M. L. *J. geophys. Res.* **75**, 7888-7915 (1971).

Fault location by radon and mercury detection at an active volcano in Nicaragua

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The Masaya Caldera Complex, Nicaragua, with the active crater, Santiago (Fig. 1), is the site of present-day basaltic volcanism^{1,2}. Some of the faults that bound the caldera and lie within it can be located geologically; others are postulated. A possible older caldera rim lies to the south of the present margin. To investigate whether one can identify faults in this environment of active volcanism, concentrations of radon and mercury in the regolith were determined at 232 selected points in and adjacent to the caldera. Higher concentrations of Hg⁰ or Rn or both occurred over 75% of the known structures studied. Some hitherto suspected structures were confirmed while others were not. Lack of a chemical signature may be ascribed to the absence of a structure, inappropriate sampling sites, post-fault lava cover or other sealing of the structure. We show here how the use of Rn and Hg⁰ determinations has helped in structural analysis at this active volcano.

Radon was detected using α sensitive film enclosed in Track Etch Detector Cups which were buried at 40-cm depth for ~30 days. Analyses of the tracks on the film were performed by the manufacturer. Radon is reported as tracks per mm² per 30 days

(0.91 tracks per mm² per 30 days = 1 pCi l⁻¹ for the allyl deglycol carbonate detector used)³. Replicate radon analyses indicated a deviation of 10%, similar to the deviation reported by others⁴⁻⁶.

Mercury was determined, using a gold film mercury detector (Jerome Instrument Corp., Model 301), in the <0.5 ϕ (0.17 mm) size fraction of the regolith collected from 40 cm depth at each of the Rn locations. The 5% deviation indicated by replicate analyses agrees with that of Cox and Cuff⁴.

Traverses (Fig. 1) were made⁷ approximately perpendicular to known or suspected faults or fissures, and were usually 150-300 m long with 6 or 7 samples per traverse. Seventeen traverses crossed structures for which geological evidence was strong. Sixteen other traverses crossed postulated structures.

In a previous study⁵ Rn was successfully used to delineate fault zones in a known geothermal area, but was of different scale (60-150 m cup spacing) and in different terrain than the one described here. A geothermal resources survey on the north rift zone of Haleakala volcano, Hawaii used a 1-1.5 km sample spacing⁴. Significantly elevated Hg⁰ and Rn values marked the

Table 1 Variations in Rn and Hg⁰ with nature of the regolith

Nature of regolith	Radon (tracks per mm ² per 30 days)		Hg ⁰ (p.p.b.)	
	Geometric mean	s.d.	Geometric mean	s.d.
Recent lava flow	3.3	+2.6 -1.5	21.8	+17.4 -9.7
Old flows with moderate soil cover or ashfall or scoriafall	10.1	+7.1 -4.2	25.3	+15.2 -9.5
Well developed soil, often cultivated	45.1	+94.7 -30.6	35.8	+17.9 -11.9

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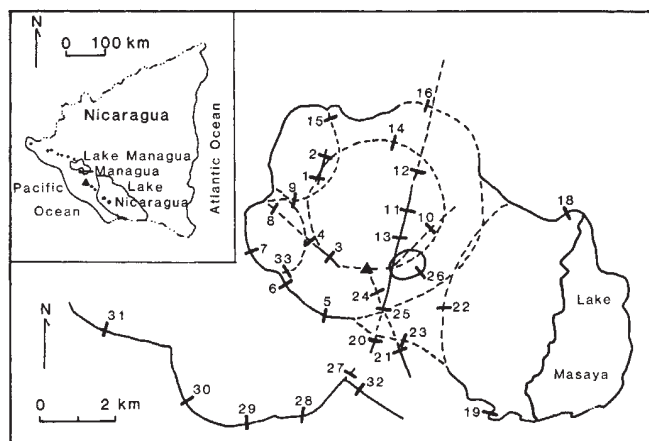


Fig. 1 Partial and simplified map of structures of Masaya Caldera Complex. Solid lines indicate location of faults or fissures known on basis of geology; dashed lines indicate location of postulated faults or fissures; bars indicate location of traverses (not to scale). Numbers of traverses are referred to in text (traverse 17, not shown, lies to north on regional fault). $\blacktriangle$, Location of Santiago crater, site of present-day activity. Inset shows location of Quaternary volcanoes ($\bullet$) and study area ($\blacktriangle$) in Nicaragua.

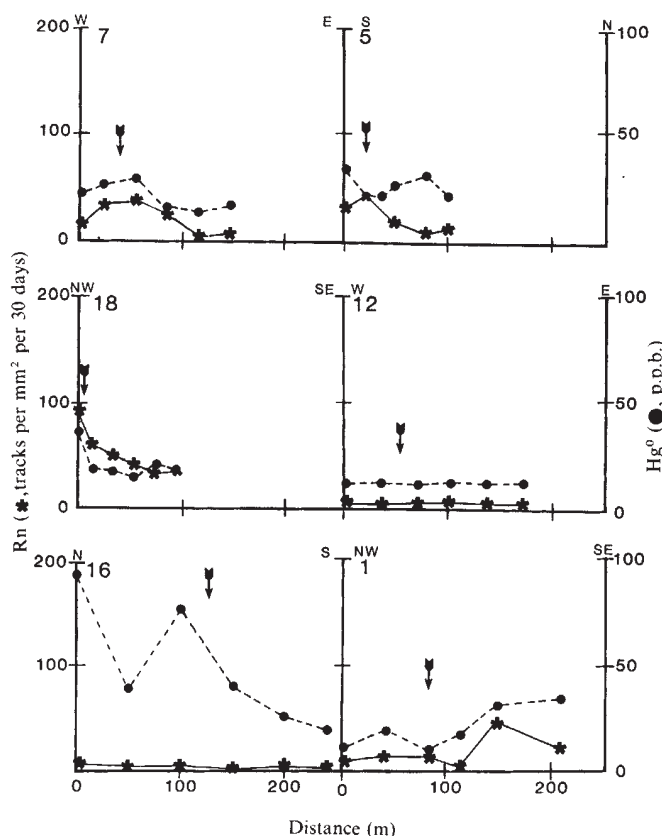


Fig. 2 Rn and Hg^0 signatures from selected traverses (numbers in upper left of each example as in Fig. 1). Arrow indicates location of fault or fissure based on field geology. Individual traverses: 7, Hg^0 and Rn peaks coincide with each other and with projected fault location (caldera fault scarp); 5, Rn and Hg^0 peaks separated by 60 m (caldera fault scarp); 18, Fault probably on the extension of the traverse (caldera fault scarp); 12, Neither Rn or Hg^0 show significant change along traverse (an attempt to locate position of fissure); 16, Hg^0 signature 2 peaks, but no significant Rn peak (hidden caldera fault scarp); 1, Hg^0 peaks and a less marked Rn peak separated by 50 m (an attempt to locate portion of a fissure).

rift zone. The survey reported here examined narrower fault zones and in much greater detail.

Data have been selected from this study to show the variation in Hg^0 and Rn signatures (Fig. 2). Radon and mercury signatures marked the caldera-bounding fault on all traverses across it (5, 6, 7, 16, 18, 19, 23) although detection of two segments where visual expression was lacking (16, 23) was uncertain. The scalloped caldera boundary suggests coalescing collapses rather than a single engulfment¹. Both Rn and Hg^0 analyses on four of the five traverses crossing presumed locations of such structures (4, 8, 9, 15, 33) indicate that these circular features may exist beneath the covering of recent basaltic lava flows. Circular distribution of active vents in the caldera may outline the trace of a collapse crater¹ but Rn and Hg^0 analyses (3, 14) do not support its existence. A third hypothesis suggests that there may be two overlapping caldera collapse features (J. Garayar, personal communication). The traverse (22) designed to test such a hypothesis did not support its presence. A proto-caldera rim, outside the present scarp, may be represented by a deeply eroded fault scarp. Rn analyses from samples on traverses across this fault (27–32) confirm its presence. Hg^0 analyses were less conclusive. Linear features cross the caldera floor, but prediction of their location, despite surface expression in some instances, met with mixed success. Of 11 traverses across faults or fissures, one (11) failed to indicate a fault, two (4, 25) indicated possible faults and the others (2, 3, 8, 10, 12, 13, 20, and 24) indicated faults in the predicted location. For the 16 traverses over known faults or fissures Rn and Hg^0 both successfully predicted 75% and for the 17 traverses over possible faults Rn successfully predicted 65% and Hg^0 76%.

Radon values (Table 1) are very much larger in areas of well developed soil, as in Hawaii⁸, but the ratio of peak to background values is greater in most localities outside the area of well-developed soil. Factors affecting radon concentration include recent lava flows acting as effective barriers to ground gas migration and the fact that the radon content in the tholeiitic lavas is lower than in the more siliceous volcanics in the areas of best soil developed outside the caldera. The high porosity of soils compared with the sparse regolith developed over recent flows allows more effective radon migration and the greater surface area enhances radon emanation. At Masaya Caldera Complex, meaningful measurement of radon in areas of fresh lava flows is feasible, despite the very low peak values.

The Hg^0 content of the fresh lava sampling sites (2, 3, 9, 11–16, 22, 23, 25) (geometric mean of 21.8 p.p.b. (part per 10⁹)) is much greater than reported⁹ from Icelandic lava, 2–9 p.p.b. Hg^0 data (Table 1) indicate less distinctive populations in the three areas defined by the type of regolith. The ratio of peak to background values is usually smaller in areas of well-developed soil than elsewhere. Secondary soil processes may be important in determining the background variation of Hg^0 , as at Long Valley California¹⁰.

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- McBirney, A. R. *J. geophys. Res.* **37**, 83–96 (1956).
- Williams, S. N. *IAVCEI Symp. Arc Volcanism*, Tokyo, 416 (1981).
- Fleischer, R. L. *et al. Tech. Inform. Ser. Rep. 79CRD263* (General Electric Schenectady, New York, 1979).
- Cox, M. E. & Cuff, K. E. *Trans. Geotherm. Resour. Council*, **4**, 451–455 (1980).
- Nielson, D. L. *Earth Sci. Lab. Rep. ESL-14* (University of Utah Research Institute, 1978).
- Gingrich, J. E., Alter, H. W. & Fischer, J. C. *8th int. Geochem. Explor. Symp.*, Hannover (1980).
- Crenshaw, W. B. thesis, Dartmouth College Hanover, New Hampshire (1982).
- Wilkening, M. H. *Science* **183**, 413–415 (1974).
- Coderre, J. A. & Steinthorsson, S. *Geochim. cosmochim. Acta* **41**, 419–424 (1977).
- Klusman, R. W. & Landress, R. A. *J. Volcan. Geotherm. Res.* **5**, 49–64 (1979).

Hidden cues in random-line stereograms

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Vernier acuity shows that the human visual system can interpolate the relative position of a feature with astonishing precision, of the order of a fraction of the distance between neighbouring photoreceptors in the fovea. The remarkable properties of this interpolation process have been the subject of many recent studies¹⁻⁹. A major question that is still unanswered is whether the process is parallel—operating automatically at all times over a large part of the visual field—or serial—operating selectively at isolated locations only when judgments in the hyperacuity range are required (as in a forced choice task). Successful fusion of random-line stereograms having breaks in the vernier acuity range has been previously interpreted¹⁰ to suggest that the interpolation process underlying hyperacuity is parallel and preliminary to stereomatching. Here we demonstrate with computer experiments that vernier cues are not needed to solve the stereomatching problem posed by these stereograms, and we provide psychophysical evidence that human stereopsis probably does not use vernier cues alone to achieve fusion of these random-line stereograms.

Julesz and Spivack's¹⁰ claim that stereo fusion of their random-line stereograms is based on vernier cues alone is apparently the best evidence for the interpolation process being parallel; Fig. 1a shows an example of their random-line stereograms. Figure 2 illustrates by a small 4×5 array of picture elements, the way in which Fig. 1a was generated. Each picture element contains a thin vertical line segment at one of two possible horizontal positions, separated by one-fifth of the picture element diameter. The random selection of the two positions to make the 4×5 array produces five vertical lines through the array with frequent small horizontal breaks. Once the left array is generated in this way, it is copied into the right array where a rectangular region of picture elements at the centre (dotted lines) is shifted horizontally by an integral number of picture element diameters (here the shift is one picture element). Thus the pattern of breaks is correlated in the left and right arrays with zero disparity around the borders and a fixed horizontal disparity in the central region. Figure 1a is the same except that the array has 100×100 picture elements and the horizontal shift is two picture elements. Similar patterns can also be made with horizontal line grids. The only obvious monocular information is the pattern of minute horizontal breaks occurring randomly in the thin vertical line grids. The stereograms clearly yield stereopsis—the centre square is seen in front of the surround—even with monocular breaks of as little as 16 arc s, which is below the threshold for resolving two lines. Note that the stereomatching problem posed by this type of stereogram is not solvable by simply measuring the position disparity between nearest line segments in the two arrays of the stereo pair. The correct matches in the central region are shifted horizontally by one or more picture elements so the nearest matches are all false targets. Thus, the matching problem can only be solved by taking into account, in some manner, the pattern of breaks in an area. For this reason Julesz and Spivack¹⁰ argued that it is necessary to detect the vernier breaks and match corresponding ones before stereo fusion.

The key question is whether vernier cues are indeed the only monocular information present in this type of stereogram. It has been suggested to us (H. Barlow and W. Richards, personal communications) that receptive fields—for example, centre-surround ganglion cells—each integrating over angular extents of several arc min might detect coarse monocular structures in such stereograms. If so, this structure alone may be sufficient to drive binocular stereomatching without need of vernier precision. Indeed, a recent theory of human stereo vision¹¹⁻¹⁴ (see also ref. 15) proposes that the two images are filtered through several, roughly bandpass, channels, each with a different centre-surround receptive field size, and that stereomatching is based mainly on zero-crossings (boundaries between regions of positive and negative response) in each of these channels. Essentially all images (for example, random-dot stereograms) when processed in this way, reveal a clear coarse structure (see for example Fig. 3.6 of ref. 13). When the random-line stereogram of Fig. 1a is filtered in the same way, coarse monocular features do appear (Fig. 1b). The underlying cause of this coarse structure is the variation in spacing between neighbouring lines. While the absolute changes in spacing are small, the percentage change in the line spacing is appreciable. For the patterns described by Julesz and Spivack, the ratio of smallest to largest line spacing is 2:3. Figure 1b shows the locations where a centre-surround operator would give positive (white) and negative (black) responses to the random-line pattern shown in Fig. 1a. The coarse structure here is well preserved by the photoreceptor sampling at intervals of 30 arc s. Interpolation between these points to increase spatial precision is unnecessary.

Many matching algorithms can successfully use the monocular features provided by the larger operators to solve the stereo correspondence problem. As an example, Fig. 1c shows the output obtained with the cooperative algorithm of Marr and Poggio^{16,17} when applied to the binary array shown in Fig. 1b. It identifies correctly the different disparities of the centre square and the surrounding background. Essentially the same results are obtained with the other stereomatching algorithms developed in our laboratory, when applied to the same filtered images. Thus these random-line stereograms can be solved without need of vernier precision.

At this point, our computer demonstration shows only that non-vernier, monocular cues are present and could be used for successful stereomatching, but the question remains of whether our visual system actually uses them instead of the vernier cues. Psychophysical experiments can be used to test these hypotheses. We have examined the differences expected between systems relying entirely on one or the other approach. As noted above, the coarse structure shown in Fig. 1b is due to the large line spacing variation in the patterns. Increasing the line spacing while maintaining a fixed break size reduces the detectability of coarse structure present in the patterns but does not affect the detectability of the vernier breaks (larger spacings should actually improve vernier acuity⁶).

Figure 3 shows a random-line stereogram having twice the line spacing as shown in Fig. 1, but with the same break size and disparity for the central region. This stereo pair is difficult to fuse when viewed at a distance which gives a vernier break size of about 15 arc s while the pair of Fig. 1a can be fused easily at that distance. These patterns were computer-generated on a high-resolution cathode ray tube and viewed stereoscopically from a distance of 4 m at which the size of the vernier breaks was ~ 15 arc s. We tested four subjects in these conditions and for all of them the stereogram of Fig. 3a was much more difficult to fuse than Fig. 1a; this is consistent with the computer simulations (compare Fig. 1c with Fig. 3c).

Other psychophysical observations, though less critical, are also consistent with the large channel hypothesis. For example, we expect that (1) random-line stereograms could be fused for very small break sizes if line spacings were made proportionally smaller—fusion would eventually be limited by the loss of contrast as the receptive field extends across more than several

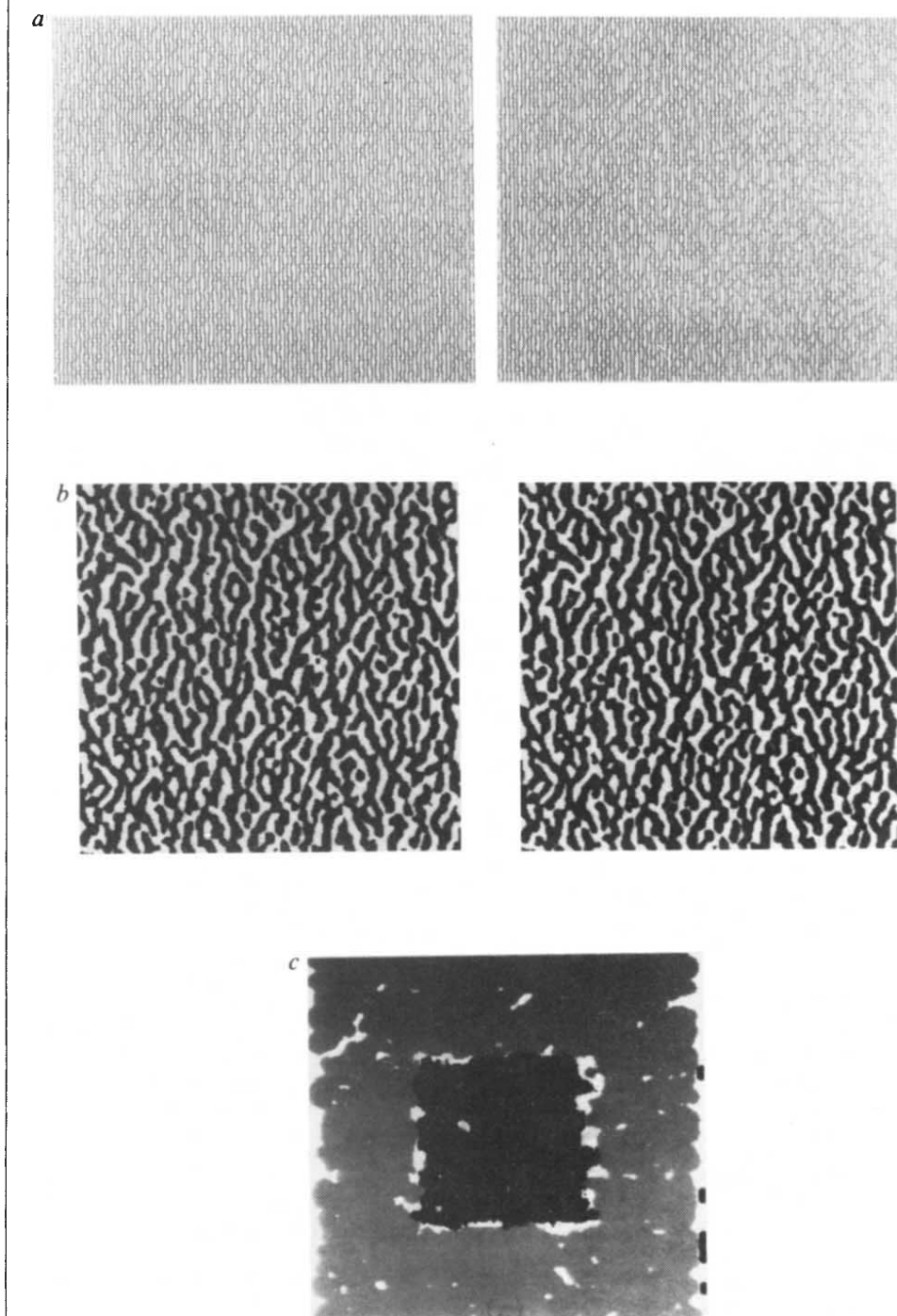


Fig. 1 *a*, Random-line stereograms of thin vertical line segments with small breaks, portraying a centre square in front of the surround. When the patterns as printed here are viewed at 1.4 m, the vernier breaks are ~ 15 arcs and the minimum line spacing is 1 arc min (the spacing and the diameter of the cones in the human fovea is ~ 30 arcs). The central square has a disparity relative to the background of 10 times the break size. *b*, The stereogram of *a* filtered through a centre-surround operator consisting of the difference of two gaussians (DOG)¹⁹. The width of the operator's centre is 16 times the break size in *a*. When *a* is viewed from a distance making the break size 15 arcs, this corresponds to what a filter with a 4-arc min centre would compute—a medium-sized channel as revealed by psychophysical experiments^{11,20,21}. Positive values are shown as white and negative as black; white (black) values would then represent the activity of the corresponding ON (OFF) centre-surround ganglion cells. The patterns in *a* were first convolved with a gaussian ($\sigma = 20$ arcs) to simulate the optics of the eye; this result was then sampled at intervals of 30 arcs before convolution with the DOG operator. *c*, The result of applying the stereomatching algorithm of Marr and Poggio¹⁶ (see legend of Fig. 3c) to the (binary) array shown in *b*. The grey levels here indicate the disparity of the matches obtained by the algorithm after five iterations, black corresponding to +6 pixels disparity (+3 arc min), and zero disparity appears as grey. White indicates non-matched pixels. The algorithm successfully extracts the correct disparity information without need of vernier interpolation.

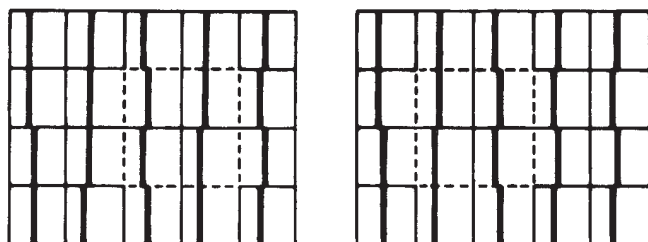
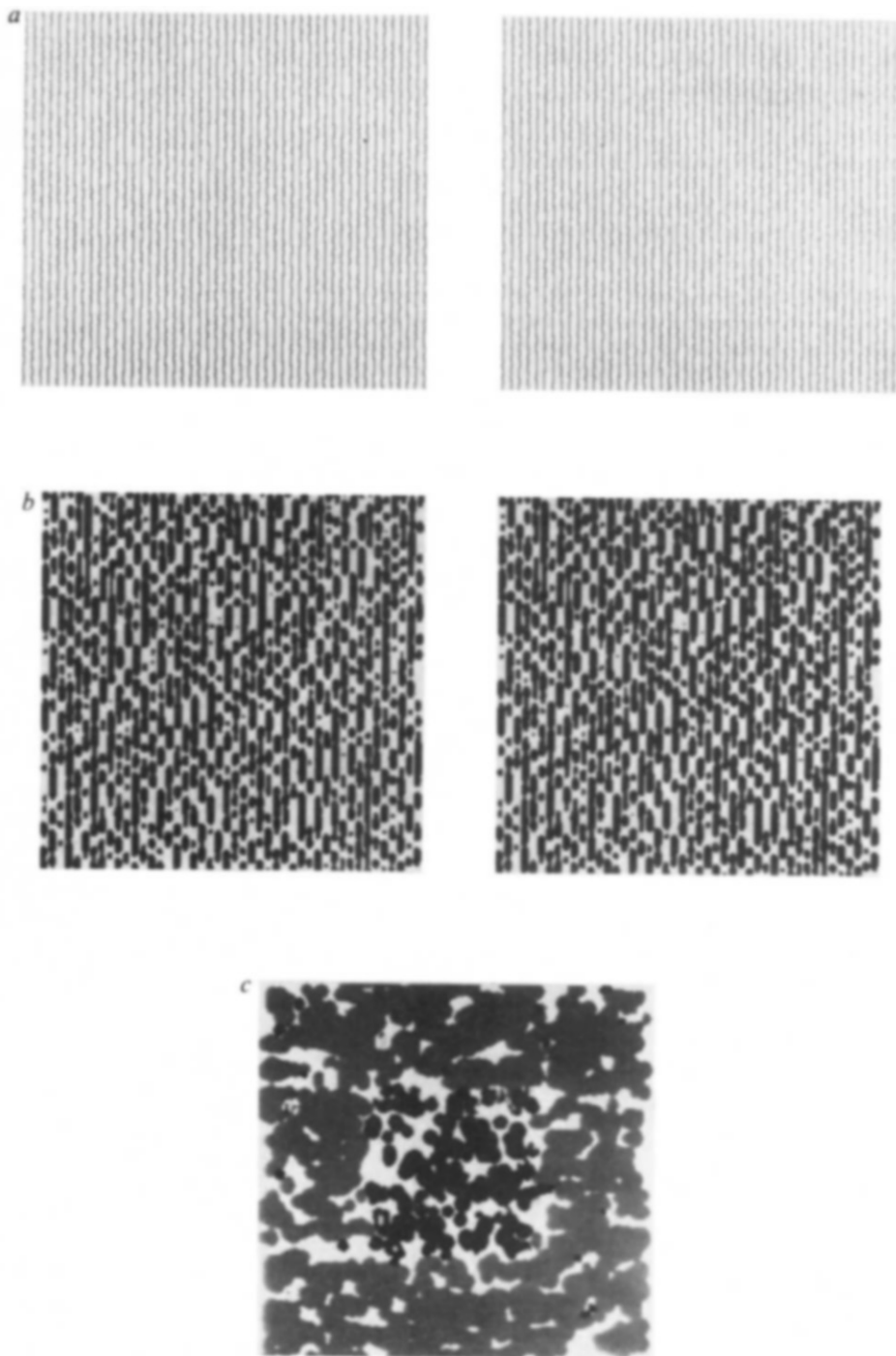


Fig. 2 Method by which the stereogram of Fig. 1a was generated (from Julesz and Spivack¹⁰).

lines; and (2) the disparity limit should be larger for horizontal random-line patterns. The regions of positive response in Fig. 1b are elongated slightly in the direction of the lines. This gives a larger average horizontal distance between vertical zero-crossings for the case of horizontal random-line patterns, and thus larger disparities can be viewed without confusion from false targets. Julesz and Spivack¹⁰ report both phenomena: fusion continuing weakly with break sizes beyond the threshold for vernier acuity, and larger disparity range for horizontal patterns.

Thus, our computational experiments establish that the stereomatching problem posed by the random-line stereograms can be solved without vernier interpolation, while the psychophysical data suggest that human perception of these

Fig. 3 *a*, A random-line stereogram with the same break size as in Fig. 1*a* but twice the line spacing. The disparity of the central square is the same as in Fig. 1. *b*, The sign of the convolution of *a* with a centre-surround operator as in Fig. 1*b*. The vertical line grid in *a* dominates the coarse structure shown here; there is much less effect from the variation in line spacing due to the pattern of breaks. *c*, The result of applying the stereomatching algorithm to the array of 3*b* after five iterations. No match is obtained (white regions) over a larger portion of the array compared with Fig. 1*c*. The algorithm used here, as in Fig. 1*c*, is the cooperative algorithm of Marr and Poggio^{16,17} operating on the sign of the stereo images after convolution with a DOG mask. Parameters are the same as described in ref. 16. The network corresponding to the algorithm is loaded by an 'and' operation on the 'binarized' convolved images. In this way the cooperative algorithm originally described for random-dot stereograms can be successfully used on stereo pairs of natural images, at several different resolutions (set by the size of the DOG mask). In Fig. 1*c* and 3*c* we have used seven disparity layers covering a total disparity range of ± 7 pixels. We have run the algorithm with different disparity ranges and also with rather different parameters, obtaining similar results.



stereograms does not rely on vernier acuity cues. The reason for this is that the elegant random-dot and random-line stereograms of Julesz¹⁸ and Julesz and Spivack¹⁰, when appropriately processed, exhibit otherwise hidden cues that simplify the stereomatching problem. (The same cues may have a significant role in human stereo vision of natural images.) The stereograms therefore cannot be used to support the hypothesis that the detection of vernier breaks is computed in parallel and made available to other later processes.

Received 21 June; accepted 27 September 1982.

1. Barlow, H. B. *Nature* **279**, 189–190 (1979).
2. Barlow, H. B. *Proc. R. Soc. B* **212**, 1–34 (1981).

3. Westheimer, G. *Am. J. Optom. physiol. Opt.* **53** (7), 362–364 (1976).
4. Westheimer, G. & Hauske, G. *Vision Res.* **15**, 1137–1141 (1975).
5. Westheimer, G. & McKee, S. P. *Vision Res.* **17a**, 89–93 (1977).
6. Westheimer, G. & McKee, S. P. *Vision Res.* **17b**, 941–947 (1977).
7. Westheimer, G. & McKee, S. P. *Invest. Ophthalmol. Vis. Sci.* **118**, 614–621 (1979).
8. Crick, F. H. C., Marr, D. & Poggio, T. in *The Cortex* (ed. Schmitt, F. O.) (MIT Press, 1981).
9. Fahle, M. & Poggio, T. *Proc. R. Soc. B* **213**, 451–477 (1981).
10. Julesz, B. & Spivack, G. J. *Science* **157**, 563–565 (1967).
11. Marr, D. & Poggio, T. *Proc. R. Soc. B* **204**, 301–328 (1979).
12. Marr, D. C. & Poggio, T. *A.I. Memo 558* (Massachusetts Institute of Technology, 1980).
13. Grimson, W. E. L. *From Images to Surfaces* (MIT Press, 1981).
14. Marr, D. *Vision* (Freeman, San Francisco, 1982).
15. Mayhew, J. E. W. & Frisby, J. P. *Artif. Intell.* **17**, 349–385 (1981).
16. Marr, D. & Poggio, T. *Science* **194**, 283–287 (1976).
17. Marr, D., Poggio, T. & Palm, G. *Biol. Cybern.* **28**, 223–239 (1978).
18. Julesz, B. *Foundations of Cyclopean Perception* (University of Chicago Press, 1971).
19. Nishihara, H. K. & Larson, N. G. *Proc. DARPA Image Understanding Workshop* 114–120 (Science Application Inc., Arlington, 1981).
20. Wilson, H. R. & Bergen, J. R. *Vision Res.* **19**, 19–32 (1979).
21. Marr, D. & Hildreth, E. *Proc. R. Soc. B* **207**, 187–217 (1980).

ON and OFF layers in the lateral geniculate nucleus of the mink

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The lateral geniculate nucleus (LGN), a major target of the optic nerve in mammals, is a laminated structure, although the pattern of lamination varies widely among different species. It has long been known that one function of the lamination is to segregate input from the two eyes. Many animals, however, have more than one layer innervated by each eye; the functional significance of these duplications has been only partially elucidated. In the mink, the principal layers innervated by the contralateral eye (layer A) and the ipsilateral eye (layer A1) are each split into two adjacent leaflets¹. We report here that the anterior leaflet of each pair contains ON-centre neurones and the posterior leaflets OFF-centre neurones.

Extracellular recordings were made from the LGN of six adult male minks (*Mustela vison*) of the brown-eyed pastel variety. The minks were anaesthetized with pentobarbital, paralysed with galamine triethiodide and mechanically respirated. The corneas were protected either with 84-D contact lenses or with silicone fluid. Standard electrophysiological techniques were used². Tungsten electrodes (tip impedance 10 M Ω at 1 kHz) were lowered vertically through the LGN, and visual responses were assessed at 50- μ m intervals. The electrodes permitted the isolation of single units (at ~40% of all recording sites) and also recorded rich multi-unit activity. Stimuli consisted of light spots and annuli for the study of centre-surround organization. In one animal, counterphased square wave gratings (0.5 or 1 cycle per deg) were also used to assess the linearity of spatial summation within receptive fields. Points of interest were marked with electrolytic lesions (3–5 μ A for 3–5 s,

electrode negative). At the end of the experiment, the animal was perfused with formol-saline. The LGN was sectioned parasagittally and stained with cresyl violet for reconstruction of electrode tracks.

The lamination of the mink LGN is illustrated in Fig. 1a, b. Its most striking feature is the pair of A laminae (A_{ant} and A_{post}), both of which receive input from the contralateral eye¹. A_{ant} is separated from the optic radiation by the perigeniculate nucleus (PGN). A_{ant} and A_{post} are separated from each other by a thin cell-sparse zone. Behind the A laminae lie the two A1 layers ($A1_{\text{ant}}$ and $A1_{\text{post}}$), both of which are innervated by the ipsilateral eye. The A1 laminae differ from the A layers in that they are thinner, less clearly separated from one another, and occupy only the ventromedial portion of the nucleus. Further posterior are a thin magnocellular layer innervated by the contralateral eye (C_m), and at least two parvocellular C layers (C_p). Aside from the sublamination of the A and A1 layers and the large size of the monocular segment, the mink LGN resembles that of the cat³.

Because of the curvature of the layers, an electrode driven vertically through the LGN will pass obliquely or tangentially through several or all of the layers. One such penetration is illustrated in Fig. 1a, b. In the C layers, mixed ON- and OFF-centre responses were obtained on stimulation of the contralateral eye. In the A sublaminae, cells were also driven by the contralateral eye, but ON-centre responses were confined to A_{ant} and OFF-centre to A_{post} . The transitions between ON- and OFF-centre responses were quite abrupt: one point of transition from ON- to OFF-centre activity was marked by a lesion (L1 in Fig. 1b). The lesion was centred on the cell-sparse zone between A_{ant} and A_{post} . A similar functional pattern was revealed in the A1 sublaminae, with the difference that the cells were driven by the ipsilateral eye. Again, ON-centre activity was confined to the anterior leaflet of the pair and OFF-centre responses to the posterior leaflet. A lesion was placed at the ON/OFF transition point (L2 in Fig. 1b). Histologically, the subdivision of layer A1 was not as distinct as that of layer A; nevertheless, the lesion lay squarely in the middle of the layer.

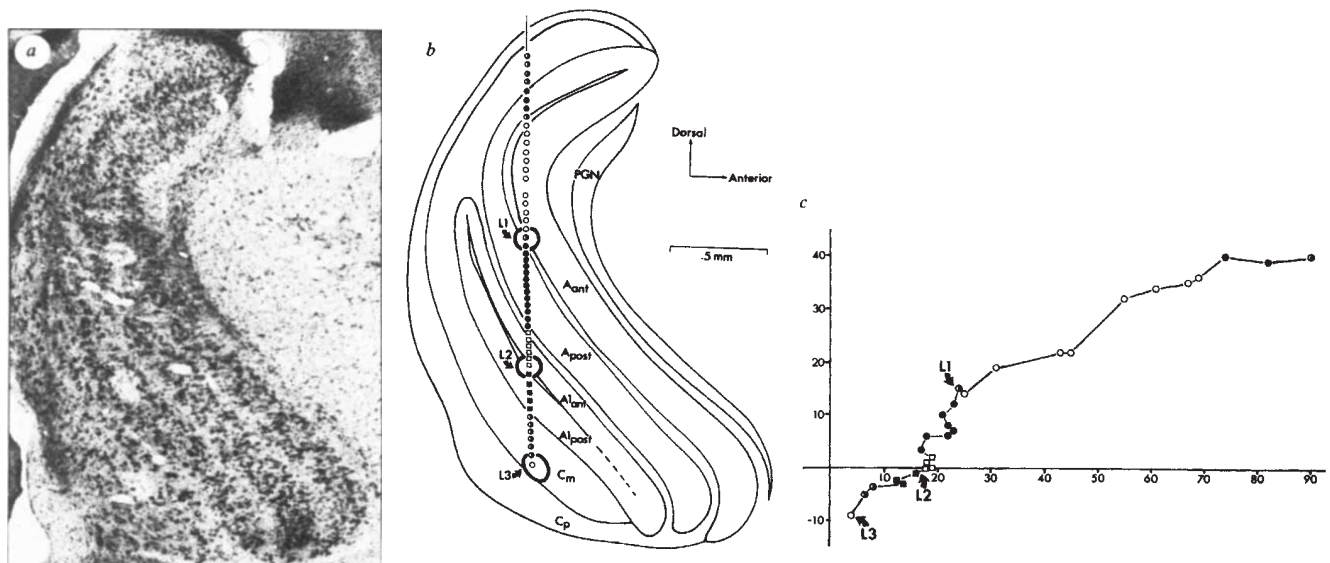


Fig. 1 Reconstruction of an electrode penetration through the left LGN of a mink. *a*, Parasagittal section (anterior to the right) stained with cresyl violet. Three electrolytic lesions made during the penetration are visible as pale spots. *b*, Key to *a*, showing the outlines of the layers, the positions of the three lesions (L1, L2, L3) and the responses obtained at 50- μ m intervals along the track (intervals were corrected for differential shrinkage). Circles indicate responses to the contralateral eye and squares to the ipsilateral eye. Open symbols indicate ON-centre responses, filled symbols indicate OFF-centre responses, and half-filled symbols indicate mixed responses. The first two lesions (L1 and L2) mark transitions from ON-centre to OFF-centre responses, and lie between the anterior and posterior leaflets of layers A and A1 respectively. The third lesion (L3) marks the end of the penetration. *c*, Progression of receptive fields across the contralateral (right) visual field, for the penetration illustrated in *a*, *b*. The axes represent the vertical and horizontal meridians. Symbols as in *b*. Each symbol is placed at the centre of the unitary or multi-unit activity recorded at a single site. Only those recording sites that gave distinct shifts in receptive field position are indicated. Fields moved downwards and medially during the penetration.

Table 1 Laminar distribution of ON- and OFF-centre responses in the mink LGN

	A _{ant}	A _{post}	A1 _{ant}	A1 _{post}	C
ON-centre	107 (100%)	0	23 (96%)	1 (7%)	24 (38%)
OFF-centre	0	62 (97%)	1 (4%)	14 (93%)	9 (14%)
Mixed	0	2 (3%)	0	0	30 (48%)

Responses at 210 recording sites in 14 penetrations are pooled. The electrodes were advanced in 50- μ m steps; a step was counted as a recording site if: (1) there was a distinct shift in receptive field position from the previous site, and (2) the laminar location of the site was subsequently determined histologically. The anterior leaflets of layers A and A1 contained predominantly ON-centre responses, the posterior leaflets OFF-centre responses, and the C layers mixed responses.

Results obtained from a further 13 penetrations in 6 animals corroborated this pattern of organization (Table 1). ON-centre responses (both single unit and multi-unit activity) were found in the anterior leaflets of A and A1 and OFF-centre responses in the posterior leaflets. This was true for both the binocular and monocular portions of the LGN. Exceptions to this pattern were found at only 4 recording sites out of a total of 210 sites in these layers. Eight ON/OFF transitions were marked with lesions; in each case, the lesion lay between the anterior and posterior leaflets of A or A1. In the C layers, on the other hand, mixed responses predominated, although we twice encountered sequences of pure ON-centre responses and must therefore leave open the possibility of some functional segregation.

Because a vertical penetration through the LGN passed roughly parallel to the layers, receptive field positions traversed a wide extent of the visual field map (Fig. 1c). Starting at the far periphery of the contralateral field at a high elevation, fields progressed downwards and medially, and ended near the vertical meridian in the lower quadrant. We have not examined the layout of the visual field map in detail, but it seems to resemble that seen in the posterior upturned portion of the cat LGN⁴.

Aside from the segregation of ON- and OFF-centre responses, receptive field properties of cells in the mink LGN also resembled those seen in the cat^{5,6}. Most cells in the A and A1 layers exhibited a typical centre-surround organization. They responded optimally to the onset or offset of a small light spot (1–2.5 deg diameter); increasing the spot size diminished the response but never reversed its sign. Responses of opposite sign could be obtained by stimulating the surround with annuli. No colour-coded responses were found when cells in layers A and A1 were tested with red, green or blue spots and annuli. In one animal, we tested 19 units in the A and A1 layers for linearity of spatial summation using a counterphased square wave grating. For some of these cells we could find a null position of the grating; others had no null position, but showed a frequency doubling⁷. Our findings suggest that, as in the cat⁶, X- and Y-like cells are intermingled in layers A and A1. We did not examine the properties of layer C cells in detail.

Our results demonstrate that the laminar organization of the mink LGN has a clear functional significance, serving to segregate cells not only by eye preference but also on the basis of ON- or OFF-centre responses. The pattern of lamination in the LGN varies considerably among mammalian species. Available physiological evidence suggests that the layers may separate different aspects of visual information in different ways. For example, the mink and cat do not seem to segregate X- and Y-like cells sharply, but such a segregation has been reported for the monkey⁸, tree shrew⁹ and bush baby¹⁰. ON/OFF segregation has been reported for the tree shrew⁹, while the macaque monkey appears to segregate ON- and OFF-centre units to some degree¹¹. Among carnivores, the cat mixes ON- and OFF-centre cells within each layer, whereas recent work on the ferret (K. Zahs and M. P. Stryker, personal communication), a close relative of the mink, suggests an organization similar to that found in the present study.

One function of laminar specialization in the LGN may be to preserve the separation of functional channels up to the

cortical level. Segregation of ON- and OFF-centre channels is already evident in the stratification of ganglion cell dendrites in the inner plexiform layer of the retina¹². Because it is thought that ON- and OFF-centre geniculate neurones are driven by retinal ganglion cells of the same sign⁵, it seems likely that axon terminals of ganglion cells terminate selectively in the appropriate leaflets of layers A or A1 in the mink LGN. Anatomical studies in several species have shown that individual layers of the LGN may project differentially to visual cortex: their terminals may occupy separate columns or layers^{13,14}. It would be interesting to examine whether the ON and OFF sublaminae of the mink LGN also project differentially to visual cortex.

Another possibility is that laminar segregation permits separate modulation of different channels of visual information. Transmission of information through the LGN is under the influence of reticular, corticofugal and intrageniculate systems^{15,16}. The segregation of ON- and OFF-centre channels into separate layers may facilitate their independent modulation, but the advantage to the mink of such an arrangement is unclear.

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1. Sanderson, K. J. *J. comp. Neurol.* **153**, 239–266 (1974).
2. Hubel, D. H. & Wiesel, T. N. *J. Physiol., Lond.* **160**, 106–154 (1962).
3. Guillery, R. W. *J. comp. Neurol.* **138**, 339–368 (1970).
4. Sanderson, K. J. *J. comp. Neurol.* **143**, 101–118 (1971).
5. Hubel, D. H. & Wiesel, T. N. *J. Physiol., Lond.* **155**, 385–398 (1961).
6. Hoffmann, K.-P., Stone, J. & Sherman, S. M. *J. Neurophysiol.* **35**, 518–531 (1972).
7. Enroth-Cugell, C. & Robson, J. C. *J. Physiol., Lond.* **187**, 517–552 (1966).
8. Dreher, B., Fukuda, Y. & Rodieck, R. W. *J. Physiol., Lond.* **258**, 433–452 (1976).
9. Conway, J., Schiller, P. H. & Misler, L. *Neurosci. Abstr.* **6**, 583 (1980).
10. Norton, T. T. & Casagrande, V. A. *J. Neurophysiol.* **47**, 715–741 (1982).
11. Schiller, P. H. & Malpeli, J. G. *J. Neurophysiol.* **41**, 788–797 (1978).
12. Nelson, R., Famiglietti, E. V. Jr & Kolb, H. *J. Neurophysiol.* **41**, 472–483 (1978).
13. Hubel, D. H. & Wiesel, T. N. *J. comp. Neurol.* **146**, 421–450 (1972).
14. Harting, J. K., Diamond, I. T. & Hall, W. C. *J. comp. Neurol.* **150**, 393–440 (1973).
15. Singer, W. *Physiol. Rev.* **57**, 386–420 (1977).
16. Dubin, M. W. & Cleland, B. G. *J. Neurophysiol.* **40**, 410–427 (1977).

Na⁺–K⁺–Cl[–] co-transport in the intestine of a marine teleost

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A key element in the absorption of salt and water by various epithelia is the presence, in the apical or brush border membrane, of a mechanism for directly coupling the inward movements of Na⁺ and Cl[–] (ref. 1). In this way the downhill gradient for Na⁺ from lumen to cell, which is maintained by the cell's Na⁺/K⁺ pump, can provide the energy for cellular accumulation and transepithelial transport of Cl[–]. A requirement for K⁺ at the serosal surface of the epithelium has long been accepted as essential to the operation of the Na⁺/K⁺ pump and therefore to salt absorption. Until very recently, however, the effect of luminal K⁺ on NaCl absorption has not been explored. We show here that luminal K⁺ stimulates NaCl absorption in the intestine of a marine teleost, the winter flounder,

Pseudopleuronectes americanus. Furthermore, K^+ uptake across the brush border membrane depends on the presence of both Na^+ and Cl^- in the bathing medium and is inhibited by furosemide, which also inhibits the coupled uptake of Na^+ and Cl^- (ref. 2). Thus, flounder intestine seems to have a $Na^+-K^+-Cl^-$ co-transport system similar to that demonstrated in erythrocytes³⁻⁵, cultured tumour cells⁶, cultured kidney cells⁷ and squid giant axon⁸. Electrophysiological studies of the thick ascending limb of Henlé's loop in mammalian kidney⁹ and the diluting segment in amphibian kidney¹⁰ also indicate that luminal K^+ is required for salt absorption. Co-transport of Na^+ , K^+ and Cl^- may therefore be characteristic of a variety of cells and tissues, including salt-absorbing epithelia.

Flounder intestine is morphologically less heterogeneous than the intestines of higher vertebrates; in particular, it contains no crypts¹¹. (Possibly for this reason, it cannot be stimulated to secrete NaCl and water¹², differing in this respect from avian and mammalian intestine, both of which possess crypts.) In short-circuit conditions and in normal Ringer's solution containing 5 mM K^+ , flounder intestine absorbs both Na^+ and Cl^- (ref. 11); neither is absorbed, however, in the absence of the other¹¹. The site of direct coupling between Na^+ and Cl^- movements is the brush border membrane, and the coupling ratio is close to 1:1 (ref. 2).

We have examined the effect of a K^+ -free luminal medium on Cl^- fluxes and the effects of ion substitutions, furosemide, bumetanide and Ba^{2+} on both K^+ fluxes and the electrical potential difference across the apical membrane (Ψ_a). Unidirectional and net transepithelial ion fluxes and initial rates of uptake from the luminal solution into the epithelium (unidirectional influxes) were measured using radioisotopes as described previously^{2,11}. Conventional voltage-sensing microelectrodes were used to determine Ψ_a (ref. 13). K^+ transport was evaluated by measuring ^{86}Rb fluxes in tissues preincubated in K^+ -free, Rb-containing Ringer's. Preliminary studies indicated that transepithelial fluxes of 5 mM ^{42}K and ^{86}Rb across flounder intestine are identical and that the transepithelial potential difference (Ψ_t), short-circuit current (I_{sc}) and Ψ_a are equally well maintained whether Rb-Ringer's or K^+ -Ringer's is used^{14,15}.

Table 1 shows the effects of Ba^{2+} and furosemide on transepithelial Rb fluxes. In their absence, short-circuited flounder intestine secretes Rb; This seems to be due to active uptake across the basolateral membrane by the Na^+-K^+ pump and diffusional exit across the apical membrane¹³. When Ba^{2+} is added to the luminal bathing medium, the direction of Rb transport reverses from secretion to absorption. Consistent with its effect on K^+ channels in several epithelia¹⁶⁻¹⁸, Ba^{2+} blocks an apical membrane K^+ channel in flounder intestine^{14,15}. When furosemide is then added to the luminal bathing medium, the Ba^{2+} -stimulated Rb absorption ceases.

Table 2 demonstrates that this inhibitory action of furosemide is exerted on a K^+ uptake process in the brush border membrane. Other characteristics of this process are also shown in Table 2: (1) Rb influx is reduced by 80% by furosemide so that, at most, 20% is paracellular. (2) Cl^- removal reduces Rb influx to the same level as does furosemide. (3) Na^+ removal

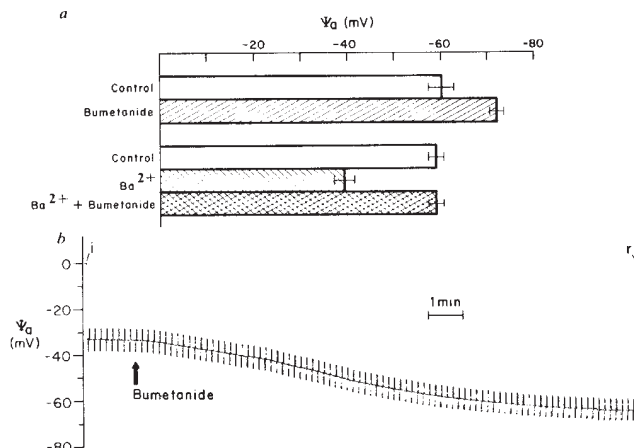


Fig. 1 Effect of bumetanide on the electrical potential difference across the apical membrane (Ψ_a). *a*, Bumetanide (10^{-4} M) was added to the mucosal solution in the presence ($n = 14$) or absence ($n = 8$) of mucosal Ba^{2+} (2.0 mM). Brackets indicate ± 1 s.e. Note the depolarization of Ψ_a by Ba^{2+} , due to blockage of apical K^+ conductance, and hyperpolarization of Ψ_a by bumetanide in the presence or absence of Ba^{2+} . Similar results were obtained with Cl^- -free mucosal solutions (see text). At least four separate recordings of Ψ_a were obtained in each experimental condition, for each tissue. *b*, Single recording of Ψ_a obtained in the presence of mucosal Ba^{2+} (2.0 mM) showing the hyperpolarization induced by addition of bumetanide (arrow) to the mucosal solution. *i*, Impalement of cell; *r*, electrode retracted. The deflections in Ψ_a result from passage of bipolar, constant-current pulses across the tissue for determination of fractional apical membrane resistance.

reduces Rb influx to the same level as does Cl^- removal. (4) Rb influx is 6–10 times greater than the unidirectional transepithelial Rb flux from mucosa $\rightarrow$ serosa, suggesting that K^+ transport across the basolateral membrane is normally rate-limiting in transepithelial K^+ absorption. (5) Ba^{2+} , despite its profound effect on transepithelial Rb fluxes, has no discernible effect on Rb influx. This surprising observation suggests that ^{86}Rb entry into the cell via the apical membrane K^+ channel is normally small. This channel, therefore, seems to strongly rectify Rb movements. Similar rectification of K^+ transport has been demonstrated in turtle colon¹⁹ and in excitable tissues²⁰.

The data in Table 2 suggest that K^+ influx across the brush border is linked to the influxes of both Na^+ and Cl^- . To determine the influence of luminal K^+ on Cl^- transport, transepithelial Cl^- fluxes and Cl^- influxes were measured in the presence and absence of luminal K^+ (Table 3). Removing luminal K^+ (with serosal K^+ maintained at 5 mM) reduced net Cl^- flux by 60% (Table 3*a*) and also reduced Cl^- influx (Table 3*b*); indeed, the furosemide-sensitive portion of the Cl^- influx was reduced by 70%. Although not directly measured, Na^+ influx is almost certainly also reduced by removing K^+ from the lumen as K^+ influx requires luminal Na^+ (Table 2) and because furosemide reduces Na^+ influxes² as well as Cl^- and K^+ influxes. The furosemide-inhibitable Cl^- influx remaining

Table 1 Effects of luminal Ba^{2+} and furosemide on transepithelial Rb fluxes across short-circuited flounder intestinal mucosa

	J_{ms}	J_{sm}	J_{net}	I_{sc}	G
Control (9)	0.34 ± 0.05	1.04 ± 0.09	-0.70 ± 0.09	-3.58 ± 0.22	26.5 ± 1.1
Ba^{2+} (9)	1.09 ± 0.10	0.48 ± 0.10	$+0.63 \pm 0.09$	-0.82 ± 0.26	21.2 ± 1.8
Ba^{2+} + furosemide (4)	0.50 ± 0.02	0.48 ± 0.06	$+0.02 \pm 0.08$	-0.21 ± 0.38	23.8 ± 2.2

Values shown are mean $\pm$ s.e. for (n) experiments. J_{ms} and J_{sm} are unidirectional fluxes from mucosa (m) $\rightarrow$ serosa (s) and s $\rightarrow$ m respectively; $J_{net} = J_{ms} - J_{sm}$. Rb fluxes and short-circuit current (I_{sc}) are in μ equiv. $h^{-1} cm^{-2}$ and conductances (G) in $mS cm^{-2}$. Intestine stripped of muscularis and mounted in chambers was bathed in K^+ -free, Rb-containing Ringer's. The medium was buffered to pH 8.0 with 5 mM *N*-2-hydroxyethyl piperazine sulphonic acid and also contained (in mM): NaCl, 170; RbNO₃, 5; CaCl₂, 1.25; MgCl₂, 1.1; Na₂HPO₄, 3; D-glucose, 20. It was maintained at 15 °C and continuously bubbled with room air. Flux measurements were begun 45–60 min after adding ^{86}Rb and 15 min after adding Ba^{2+} (to 5 mM) or furosemide (to 0.4 mM) to the luminal side.

Table 2 Effects of furosemide, Ba^{2+} and Cl^- and Na^+ replacements on lumen-to-epithelium influx of Rb

	Rb influx, $\mu\text{equiv. h}^{-1} \text{cm}^{-2}$	
	-Furosemide	+Furosemide
Effect of Ba^{2+} (4)		
Control	2.13 ± 0.32	0.48 ± 0.16
Ba^{2+}	2.39 ± 0.27	0.38 ± 0.06
Effect of Cl^- replacement (4)		
Control	3.30 ± 0.18	0.70 ± 0.12
Cl^- -free	0.64 ± 0.09	0.71 ± 0.15
Effect of Na^+ replacement (8)		
Control	2.80 ± 0.24	—
Na^+ -free	0.69 ± 0.12	—

Values shown are ± 1 s.e. for (*n*) experiments. Control tissues were preincubated in the Rb-containing Ringer's solution described in Table 1. Where indicated, Cl^- was replaced by gluconate and Na^+ by either *N*-methyl-D-glucamine or tetraethylammonium. After 60 min, ^{86}Rb was added and its influx determined over 90 s (Rb uptake is linear with time for at least 120 s). Tissues were short-circuited during the influx measurements. The influx technique is described in detail elsewhere². Ba^{2+} (to 5 mM) and furosemide (to 0.4 mM) were added to the luminal side 15–20 min before adding ^{86}Rb .

after K^+ removal from the luminal bathing medium may result from the persistence of small amounts of K^+ in the unstirred layer at the brush border surface, as the enterocytes normally secrete K^+ .

The coupling stoichiometry of the brush border $\text{Na}^+/\text{K}^+/\text{Cl}^-$ co-transport process remains to be accurately determined. Tables 2 and 3 suggest more than one Cl^- per K^+ . Our previous determinations of furosemide-inhibitable Na^+ and Cl^- influxes yield a coupling ratio (± 1 s.e.) of 1.3 ± 0.4 Na^+ per Cl^- , which does not differ significantly from 1:1 (ref. 2). These estimates of transport stoichiometry, although admittedly inexact, suggest that in flounder intestine the brush border co-transport process is electrogenic, translocating more cations than anions.

Transport-related changes in Ψ_a are consistent with these kinetic data. Inhibition of transport by addition of the furosemide analogue, bumetanide, hyperpolarized Ψ_a by 12 ± 2 mV (Fig. 1a). Similarly, complete replacement of luminal Cl^- with gluconate hyperpolarized Ψ_a by 14 ± 5 mV (*n* = 3). Cl^- replacement would be expected to depolarize Ψ_a if significant Cl^- conductance were present in the apical membrane. Finally, complete replacement of luminal Na^+ with *N*-methyl-D-glucamine hyperpolarized Ψ_a by 15 mV (mean of two experiments). All these changes are consistent with inhibition of a positively charged co-transporter. Similar hyperpolarizations have been reported for two other epithelia exhibiting K^+ -dependent NaCl co-transport: the thick ascending limb of Henle's loop in mammalian kidney⁹ and the diluting segment in amphibian kidney¹⁰.

Table 3 Dependence of Cl^- fluxes on luminal K^+

a Transepithelial fluxes (9)					
$[\text{K}^+]_{\text{m}}$	J_{ms}	J_{sm}	J_{net}	I_{sc}	
5 mM	6.17 ± 0.48	2.63 ± 0.33	3.54 ± 0.32	-1.78 ± 0.16	
0	$3.93 \pm 0.62^*$	2.49 ± 0.44	$1.44 \pm 0.56^*$	$-0.93 \pm 0.10^*$	
b Influxes (9)					
$[\text{K}^+]_{\text{m}}$	-Furosemide	+Furosemide	Δ		
5 mM	15.83 ± 2.09	8.20 ± 1.75	7.63 ± 0.80		
0	$12.24 \pm 1.29^\dagger$	9.97 ± 1.42	$2.27 \pm 0.56^\dagger$		

Values shown are mean $\mu\text{equiv. h}^{-1} \text{cm}^{-2} \pm 1$ s.e. for (*n*) experiments. The medium was the same as that described in Table 1 except that KCl replaced RbNO_3 . Serosal K^+ concentration was always 5 mM. $[\text{K}^+]_{\text{lm}}$, luminal K^+ concentration. The net Cl^- flux in *a* is about one-third less than that usually measured^{2,11,12}. Cl^- influx was measured with ^{36}Cl over 35 s. Furosemide was added (to 0.4 mM) to the luminal side 15 min before influx measurements. See Tables 1 and 2 for further details. Less than control: * $P < 0.05$; $^\dagger P < 0.01$.

The hyperpolarization of Ψ_a observed with inhibition of co-transport was even larger in the presence of Ba^{2+} . Addition of Ba^{2+} to the luminal solution depolarized Ψ_a (Figure 1a) and increased the fractional apical membrane resistance ($\Delta\Psi_a/\Delta\Psi_i$ in response to constant current pulses) from 0.19 ± 0.02 to 0.61 ± 0.03 ($P < 0.01$; *n* = 25). This is consistent with the inhibitory effect of Ba^{2+} on apical membrane K^+ conductance¹⁴. In the presence of Ba^{2+} , Ψ_a hyperpolarized by 20 ± 2 mV when bumetanide was added (Fig. 1a) and by 21 ± 4 mV when Cl^- was replaced by gluconate (*n* = 4). Figure 1b shows the time course ($t_{1/2}$ = 5 min) of the bumetanide-induced hyperpolarization. The time required for bumetanide to block the brush border co-transporter is unknown. Since the apical K^+ conductance channel is parallel to the co-transport pathway, Ba^{2+} would be expected to magnify the effect on Ψ_a of blocking an electrogenic co-transporter. The hyperpolarization of Ψ_a was probably not due to changes in cellular K^+ or Cl^- activities that might have resulted from inhibition of co-transport as hyperpolarization persisted when apical membrane K^+ conductance was blocked by Ba^{2+} and because significant basolateral membrane K^+ or Cl^- conductances have not been detected in ion replacement studies¹⁴. However, the possibility that there is an obligatory inverse relationship between apical membrane K^+ conductance and co-transport rate cannot be excluded. Additional studies are needed to define whether the epithelial co-transport process (in contrast to that of avian erythrocytes⁵) is affected by changes in membrane potential.

The results presented here suggest that what was initially conceived to be an epithelial membrane $\text{Na}^+/\text{K}^+/\text{Cl}^-$ co-transport is in fact a $\text{Na}^+/\text{K}^+/\text{Cl}^-$ co-transport; the involvement of this triple co-transport in cell homeostasis is largely unexplored. It may facilitate regulation of cell volume in non-epithelial cells and regulation of fluid transport across low resistance epithelia. As long as the basolateral membrane Na^+/K^+ pump operates normally, Na^+ and Cl^- activities in the epithelial cell are less than their extracellular activities. In an electrically neutral co-transport process involving only Na^+ and Cl^- , entry is in the direction of the chemical potential differences for both ions and net transport could only be regulated by modifying co-transport capacity through the action of intracellular mediators such as Ca^{2+} or cyclic nucleotides. If K^+ also participates in the co-transport process, however, the outwardly directed K^+ gradient could, in principle, assist in regulation of net Na^+ and Cl^- entry. Conditions that might alter net flux through the co-transport pathway, including its voltage-dependence and affinities for the transported ions, remain to be defined.

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- Frizzell, R. A., Field, M. & Schultz, S. G. *Am. J. Physiol.* **236**, F1–F8 (1979).
- Frizzell, R. A., Smith, P. L., Vosburgh, E. & Field, M. *J. Membrane Biol.* **55**, 157–163 (1980).
- Dunham, P., Stewart, G. W. & Ellory, J. C. *Proc. natn. Acad. Sci. U.S.A.* **77**, 1711–1715 (1980).
- Palfrey, H. C. & Greengard, P. *Ann. N.Y. Acad. Sci.* **373**, 291–308 (1981).
- Haas, M., Schmidt, W. F. & McManus, T. J. *J. gen. Physiol.* **80**, 125–147 (1982).
- Geck, P., Pietrzyk, C., Burchkhardt, B. C., Pfeiffer, B. & Heintz, E. *Biochim. biophys. Acta* **600**, 432–447 (1980).
- McRoberts, J. A., Erlinger, S., Rindler, M. J. & Saier, M. H. *J. biol. Chem.* **257**, 2260–2266 (1982).
- Russell, J. M. *Biophys. J.* **33**, 40a (1981).
- Greger, R. & Schlatter, E. *Pflügers Arch. ges. Physiol.* **392**, 92–94 (1981).
- Sackin, H., Morgunov, N. & Boulpaep, E. L. *Fedn Proc.* **41**, 1495 (1982).
- Field, M., Karnaky, K. J. Jr, Smith, P. L., Bolton, J. E. & Kinter, W. B. *J. Membrane Biol.* **41**, 265–193 (1978).
- Field, M., Smith, P. L. & Bolton, J. E. *J. Membrane Biol.* **55**, 157–163 (1980).
- Duffey, M. E., Thompson, S. W., Frizzell, R. A. & Schultz, S. G. *J. Membrane Biol.* **50**, 331–341 (1979).
- Stewart, C. P. *et al. Fedn Proc.* **40**, 362 (1981).
- Krasny, E. J. Jr, Halm, D. R. & Frizzell, R. A. *Fedn Proc.* **41**, 1261 (1982).
- O'Neil, R. G. *Fedn Proc.* **41**, 1006 (1982).
- Nagel, W. *Biochim. biophys. Acta* **552**, 346–357 (1979).
- Kirk, K. L., Halm, D. C. & Dawson, D. C. *Nature* **287**, 237–239 (1980).
- Kirk, K. L. & Dawson, D. C. *Fedn Proc.* **40**, 357 (1981).
- Armstrong, C. M. *Biophys. J.* **15**, 932–933 (1975).

Oocyte extracts reactivate developmentally inert *Xenopus* 5S genes in somatic nuclei

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In *Xenopus* and other amphibians, there are two major classes of 5S RNA gene¹. One class, which codes for somatic-type 5S RNA, is expressed in both oocytes and somatic cells²; the other, which is 50 times more abundant³ and which codes for oocyte-type 5S RNA, is expressed in oocytes but not in somatic cells^{2,4}. The developmentally inactivated oocyte-type genes are reactivated when erythrocyte nuclei are injected into most oocytes (activating oocytes)⁵. As the injected nuclei are induced to take on the natural activity of the host cell, the activation of oocyte-type 5S genes may represent a normal control mechanism. A special opportunity to investigate this mechanism is provided by the existence of certain individual animals whose oocytes consistently fail to activate 5S genes (non-activating oocytes). We have injected extracts of activating or non-activating oocytes, together with erythrocyte nuclei, into oocytes of each kind. We show here that the transcription of 5S genes is induced by extracts of activating oocytes (positive regulation) and is not inhibited by similar extracts of non-activating oocytes. The activating factor is sensitive to protease and heat treatment, suggesting that a protein is involved.

The procedures for preparing oocyte extracts and somatic nuclei from erythrocytes^{5,6} are given in Fig. 1 legend. Somatic nuclei from mature erythrocytes were injected, either alone or after mixing with extracts prepared from either activating or non-activating oocytes, into the germinal vesicles of both types of oocyte. The oocytes were then injected with 1 µCi [α -³²P]GTP and incubated at 19 °C for 36 h⁵. Labelled transcripts were extracted and electrophoresed on non-denaturing 17% polyacrylamide gels to differentiate between somatic- and oocyte-type 5S RNA⁵.

The results are shown in Fig. 1. As found previously⁵, the oocyte 5S genes are transcribed in nuclei injected into activating oocytes (Fig. 1, lanes 3–5), but not in those injected into non-activating oocytes (lanes 1 and 2). When somatic nuclei were mixed with extracts of activating oocytes and injected into non-activating oocytes, the previously inactive oocyte-type 5S RNA genes in the somatic nuclei were transcribed about as efficiently as when such nuclei were injected into activating oocytes (Fig. 1, lanes 6 and 9). Thus, activating extracts have a strongly positive effect. On the other hand, extracts from non-activating oocytes injected with somatic nuclei into activating oocytes did not diminish the expression of the oocyte-type 5S RNA genes (Fig. 1, lanes 14 and 15). In addition, activating extracts mixed with non-activating extracts in a 1:1 ratio and injected with somatic nuclei into non-activating oocytes, continue to induce efficient transcription of oocyte-type 5S genes (data not shown). Thus, non-activating oocytes or extracts prepared from these oocytes do not contain inhibitors or nucleases specific for the oocyte-type 5S genes or their products. Extracts from activating or non-activating oocytes had no effect when injected into the same kind of oocyte (Fig. 1, lanes 10–13). In all injected oocytes, the somatic-type 5S genes of the somatic nuclei were transcribed efficiently (see Fig. 1); this serves as an internal control of the viability of the oocytes and their ability to transcribe the injected 5S genes. As activating extracts exert their influence in non-activating oocytes and not vice versa, it seems that at least one factor controlling the reactivation of oocyte 5S genes regulates 5S transcription in a positive fashion.

We have analysed the activating extract by incubating it at 70 °C for 3 min, or by incubating it with the protease papain. Somatic nuclei were mixed with the heated extract and injected into oocytes, as described above, and RNA was extracted and analysed (Fig. 1, lane 16). The oocyte 5S genes were not activated, indicating that at least one component of the activating factor is heat-labile. Activating extracts were also protease-treated by incubating with papain and neutralizing with antipain as described in Fig. 2 legend, before being mixed with somatic nuclei and injected into the germinal vesicles of non-activating oocytes. Activating potential was destroyed by the protease (Fig. 2, compare lanes 12–14 with lanes 9–11). However, when untreated activating extract was added back to the papain-antipain-treated extract, and injected as above, the oocyte-type 5S genes were transcribed (Fig. 2, lanes 15–17). Somatic nuclei mixed with activating extracts that had been pre-incubated in papain digestion buffer alone were also injected into non-activating oocytes as a further control for 5S transcription. In these conditions, the oocyte-type 5S RNA genes are transcribed (Fig. 2, lanes 18–20).

As the activating factor is heat-labile and protease-sensitive, it probably consists of one or more proteins, which act positively to activate 5S transcription. It has previously been shown that

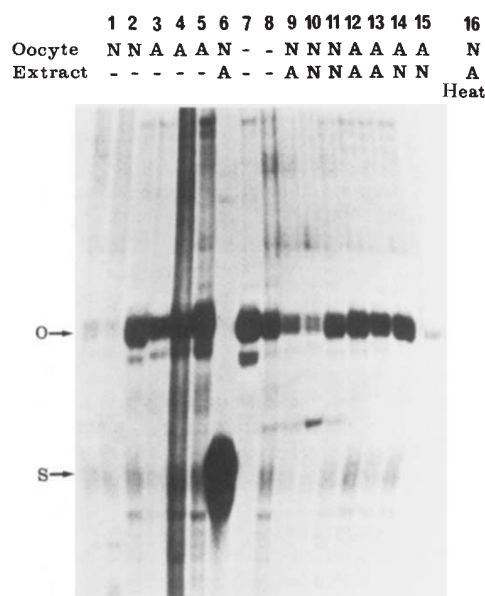


Fig. 1 Transcription of 5S RNA genes with activating and non-activating oocyte extracts. The extracts were prepared as follows: 200 single oocytes were isolated free of connective tissue, washed twice in distilled water, all excess fluid removed, then gently homogenized in a glass-glass dounce homogenizer. The extract was then centrifuged in an Eppendorf microfuge at 10,000g for 10 min at 4 °C, the supernatant was re-centrifuged at 10,000g for 15 min and the supernatant used. Somatic nuclei were prepared by lysolecithin treatment of mature erythrocytes⁶. Extracts were diluted 1:1 with somatic nuclei suspended in injection medium, just before injection into oocytes. Oocytes were injected, aiming for the germinal vesicle, with the relevant extract. After 4 h, 1 µCi [α -³²P]GTP was injected into the cytoplasm of each oocyte. After incubating at 19 °C for 36 h, labelled transcripts were extracted and run on a non-denaturing 17% polyacrylamide gel⁵. Each lane contains half the RNA from one oocyte. All the oocytes shown received an injection of erythrocyte nuclei. For each lane, the type of oocyte used and the source of extract injected (if any) is indicated: N, A, oocytes or extracts from non-activating or activating females, respectively. In lane 16, the activating extract was heated at 70 °C for 3 min. Lanes 7 and 8 are markers showing somatic- and oocyte-type 5S RNA, respectively (O and S at the left of the figure). The slight increase in oocyte-type 5S RNA in lanes 10 and 11 relative to lanes 1 and 2 is not always observed, and is small compared to the increase in lanes 6 and 9. It is enhanced by the long autoradiographic exposure used in these experiments.

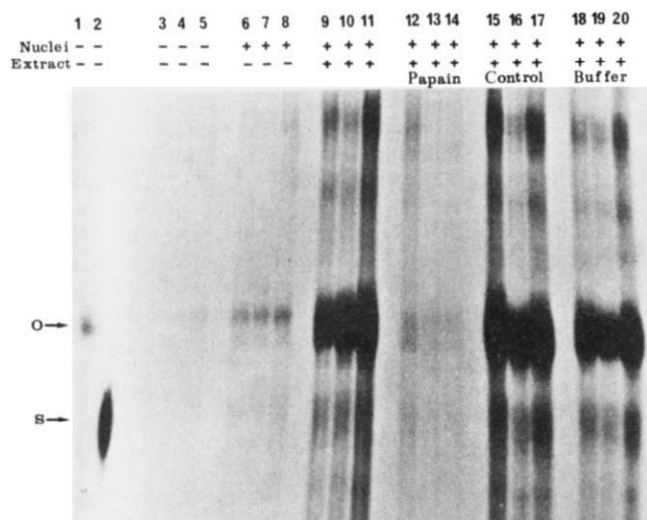


Fig. 2 Transcription of 5S RNA genes injected with activating extracts treated with protease. Oocyte extracts and somatic nuclei were prepared as described in Fig. 1 legend. All injections were into oocytes of non-activating females. Oocytes were injected with the samples and 4 h later with $1 \mu\text{Ci}[\alpha\text{-}^{32}\text{P}]\text{GTP}$. Labelled transcripts were extracted and analysed⁵. Lanes 1 and 2 contain marker 5S RNAs (O, oocyte-type; S, somatic-type). Lanes 3–20 were either injected (+) or not injected (–), with erythrocyte nuclei and/or with an extract of activating oocytes, as indicated. Lanes 3–5 represent endogenous transcription. Lanes 12–14, injection of somatic nuclei mixed with activating extract, which had been pre-incubated with 0.8 mg ml^{-1} papain (Sigma) at 20°C for 1 h, followed by neutralization with 1.25 mg ml^{-1} antipain (Sigma) for 10 min at 20°C . Lanes 15–17, injection of somatic nuclei mixed with a 1:1 ratio of papain–antipain-treated extracts (as in lanes 12–14) and untreated activating extracts. Lanes 18–20, injection of somatic nuclei mixed with activating extract and papain buffer (without papain) which had been incubated together at 20°C for 1 h.

at least two proteins in addition to RNA polymerase III are required for 5S RNA transcription^{7,8}. Only one of these, transcription factor IIIA (TFIIIA), has been purified⁷. However, TFIIIA alone does not account for the activity in activating extracts since injection of somatic nuclei plus purified TFIIIA into non-activating oocytes is not sufficient to reactivate the oocyte 5S genes⁵. Whether the activating factor acts independently of, or in conjunction with, TFIIIA has yet to be elucidated. The factor functions in the absence of DNA replication, since the mature erythrocytes used as a source of somatic nuclei represent a terminal cell type in which no DNA replication occurs⁹ and oocytes do not induce DNA synthesis in injected nuclei¹⁰.

The activating and non-activating oocytes used in these experiments represent the extremes of a continuous range of activating potential. All oocytes have sufficient activating factor to ensure the expression of their own oocyte-type 5S genes (unpublished result). However, it seems that only some have an excess sufficient to activate the extra 5S genes from several hundred injected somatic nuclei. The fact that all oocytes of a given female maintain indefinitely the same activating or non-activating property has allowed us to make use of this variation for the experiments described.

The regulation of gene expression, on the molecular level, is not understood for any eukaryotic gene. We have shown for a particular gene system, the oocyte-type 5S RNA genes, that specific, although so far unidentified, protein factors can regulate transcription in a positive fashion. We are now attempting to purify these factors.

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1. Brown, D. D. & Sugimoto, K. *Cold Spring Harb. Symp. quant. Biol.* **38**, 501–505 (1973).
2. Ford, P. J. & Southern, E. M. *Nature new Biol.* **241**, 7–12 (1973).
3. Peterson, R. C., Doering, J. L. & Brown, D. D. *Cell* **20**, 131–141 (1980).
4. Wegnez, M., Monier, R. & Denis, H. *FEBS Lett.* **25**, 13–20 (1972).
5. Korn, L. J. & Gurdon, J. B. *Nature* **289**, 461–465 (1981).
6. Gurdon, J. B. *J. Embryol. exp. Morph.* **36**, 523–540 (1976).
7. Engelke, D. R., Ng, S. Y., Shastri, B. & Roeder, R. G. *Cell* **19**, 717–728 (1980).
8. Roeder, R. G. *et al.* *ICN–UCLA Symp.* **14**, 521–540 (1979).
9. Thomas, N. & Maclean, N. *J. Cell Sci.* **19**, 509–520 (1975).
10. Gurdon, J. B. *Proc. natn. Acad. Sci. U.S.A.* **58**, 545–552 (1967).

T-cell hybridomas which produce B lymphocyte replication factors only

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B lymphocytes stimulated with BRMF (B-cell replication and maturation factor) expand clonally and mature into immunoglobulin (Ig)-secreting cells. BRMF may be a single or separate factors (BRF, BMF), capable of inducing replication and maturation in B cells^{1–3}. Experimental evidence from others^{4,5} suggests that BRF and BMF may be distinct molecular entities. We now find that some hybrids of concanavalin A (Con A)-activated splenic T cells and the thymoma BW5147 produce BRMF on stimulation with Con A, but do not produce BMF, T-cell growth factor (TCGF), T-cell replacing factor (TRF) or factors stimulating myeloid or erythroid colonies (CSF). Thus BRF and BMF are distinct activities. BRF produced by the T-cell hybridoma A32-26 appears in two major forms with molecular weights of 15,000–20,000 and 80,000–90,000.

BRMFs are made in interactions of helper T cells, Ia-compatible accessory cells and antigen^{6–17}. In the absence of antigen and helper T cells, resting B cells respond to BRMF by polyclonal maturation without replication³. B-cell maturation-inducing activity can be quantitated in plaque assays as the number of immunoglobulin-secreting cells (plaque-forming colonies, PFC)¹⁸. In order to replicate in response to BRMF, resting B cells must be raised^{3,19} to the 'excited' state, either by concomitant binding of antigen and interaction of helper T cells with Ia (antigen-specific, Ia-restricted, T-dependent activation) or by mitogens, such as lipopolysaccharide (LPS)²⁰, which circumvent binding to immunoglobulin and Ia (polyclonal, Ia-unrestricted, T-independent activation)⁸. Restimulation of LPS-activated B-blasts has provided a convenient assay for replication-stimulating activity^{1,21}. All previous BRMF preparations, as well as LPS, have also been found to induce further maturation to immunoglobulin-secreting PFC.

Cloned, antigen-specific helper T cells, as well as T-cell hybridomas, have been shown to produce several factors, including T-cell growth factor (TCGF, IL-2), BRMF, TRF and factors stimulating erythroid and myeloid colony formation (CSF) (refs 22, 23). Many of these lines have been found to produce more than one of these factors at the same time.

In an attempt to find T-cell hybridomas producing only one of these activities, we have fused BW5147 thymoma cells with activated spleen cells. (The thymoma BW5147 alone does not produce any of the activities tested.) Of 150 cloned hybridomas obtained in one fusion, 15 produced considerable quantities of factors when stimulated with Con A. Six produced TCGF, BRMF and CSF, and three produced TCGF and BRMF and no CSF. Two T-hybridoma produced only CSF and four others produced only BRMF. One of the T-cell hybridomas obtained in a similar fusion by Köhler *et al.*²⁴, called A32-26, produced also only BRMF (Table 1). This hybridoma which had already been recloned and which produced the highest levels of BRMF, was selected for further studies.

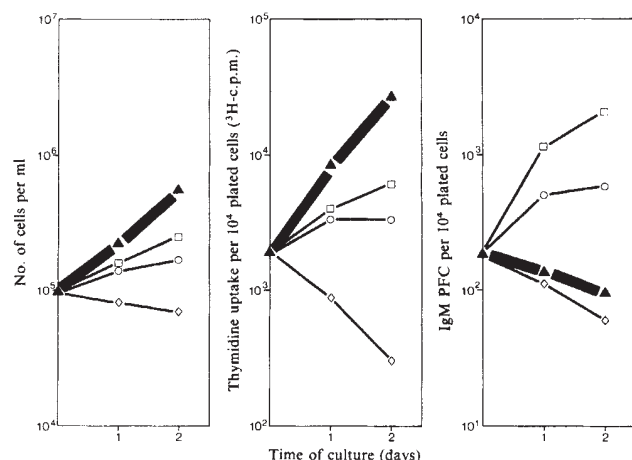


Fig. 1 Replication of LPS-activated B cell blasts (§, Table 1) in the presence of factors produced by Con A-stimulated A32-26 T hybridoma cells (*, Table 1) (—▲—), of factors produced by Con A-stimulated rat spleen cells (†, Table 1) (—□—), of LPS (50 $\mu\text{g ml}^{-1}$) (—○—), or in the absence of any added factors (—◇—). Replication was measured either by cell counts (left) or by uptake of radioactive thymidine (§ and §, Table 1) (centre). Maturation was measured as the increase in the number of IgM PFC in culture (right). IgG- and IgA-secreting PFC (not shown in the figure) behaved as the IgM PFC.

First we investigated whether Con A-stimulated A32-26 T hybridoma cells released both replication-stimulating and maturation-inducing factors. For controls the mitogen LPS and factors produced by the interaction of cloned helper cells, antigen and histocompatible macrophages²⁵ were used. LPS-activated B-blasts could be restimulated to continued replication by A32-26 factors, LPS or factors of T-cell help, as measured either by cell counts or by the uptake of radioactive thymidine (Fig. 1). LPS and factors of T-cell help increased the number of IgM-secreting PFC but factors from A32-26 T

hybridoma did not. Replication but no increased maturation was observed over a 300-fold range of concentrations of A32-26 factors of 50–0.15% in culture. In control experiments we found that LPS-stimulation of B cells either in the presence or absence of factors from A32-26 cells developed a similar number of IgM PFC, indicating that these factors did not inhibit IgM secretion or plaque formation. We conclude that on stimulation by Con A the T hybridoma A32-26 cells produce BRF but not BMF. Judging by replication in the presence of A32-26 BRF and LPS, A32-26 BRF stimulates polyclonal activation of B-cell blasts. This polyclonal restimulation is also independent of the H-2 or immunoglobulin haplotype of the B-blasts, since B-blasts of C57BL/6J *nu/nu* mice (Ig^b, H-2^b), BALB/c *nu/nu* mice (Ig^e, H-2^d) and A/J mice (Ig^e, H-2^{k/d}) could all be restimulated. This is in line with earlier observations, that neither surface immunoglobulin nor surface Ia have to be occupied by ligands to allow BRF to stimulate B-blast through the next round of division.

The activity of helper T cells in T-dependent B-cell stimulation can be replaced by soluble factors (TRF) obtained in the interaction of helper T cells, antigen and macrophages^{8–15}. Thus, B cells of athymic *nu/nu* mice will respond to antigens such as sheep erythrocytes (SRBC) by clonal replication and maturation to secrete SRBC-specific antibody in the presence of such TRF. Factors from T hybridoma A32-26 have no activity detectable as TRF, while other media expected to contain TRF, do, indeed show activity (Table 1). We think that this is because they do not contain BMF which could induce SRBC-specific replicating B cells to cells secreting SRBC-specific antibody, detectable as PFC.

The apparent molecular weight of BRF produced by the A32-26 T hybridoma cells was estimated by gel filtration over a G100-Sephadex column. BRF is detectable in two major molecular forms, with apparent molecular weights of 80,000–90,000 and 15,000–20,000, and two minor forms with molecular weights of ~45,000 and >100,000 (Fig. 2). Both human and murine BRF have also recently been identified in other laboratories^{5,26}. Structural studies on the chemical nature of the BRF from A32-26 T hybridoma cells and from other BRF-producing hybridomas should clarify whether the different

Table 1 Proliferation of T- and B-cell blasts in media conditioned by factors from different sources

Medium conditioned with	CTL-L killer T cells†	Helper T cells*	LPS-activated B-blasts §	SRBC-specific PFC per 2×10^6 <i>nu/nu</i> C57BL/6J cultured spleen cells**	Colonies per 10^5 bone marrow cells††
	($10^{-3} \times ^3\text{H}$ c.p.m. TCGF)	thymidine uptake per 2×10^4 plated cells TCGF	BRMF	TRF	CSF
Con A-activated T-hybridoma A32-26 cells*	0, 15	0, 18	15, 0	<25	0
Helper T cells†	7, 5	11, 5	6, 0	9,500	51
LPS‡	0, 15	0, 2	10, 0	2,000	0
Con A-activated spleen cells†	15, 0	14, 0	14, 0	15,000	ND
None	0, 15	0, 15	0, 8	<25	0
Con A-activated BW 5147 cells*	0, 1	0, 1	0, 3	<25	0
Con A	0, 1	0, 1	0, 2	<25	0
WEHI-3 cells	0, 1	0, 1	ND	<25	90

* T hybridoma and BW5147 cells were stimulated at 5×10^5 cells per ml for 24 h with $5 \mu\text{g ml}^{-1}$ Con A in serum-free medium³³ in a CO_2 -incubator at 9% CO_2 in air. The conditioned media were used at 25% (except the Con A-activated spleen cell supernatants, see below), in the presence of 5mM α -methylmannoside (Sigma). All *in vitro* experiments described here were done in serum-free medium³³.

† 5×10^6 rat spleen cells per ml incubated with $5 \mu\text{g ml}^{-1}$ Con A for 24 h, supernatant medium concentrated by ammonium sulphate precipitation and partially purified by G100-Sephadex chromatography. Used at 10%.

‡ The CTL-L killer T-cell line was a gift of Dr K. Smith. 5×10^4 CTL-L cells per ml were cultured in the conditioned media for 48 h, then $1 \mu\text{Ci } ^3\text{H}$ -thymidine (2Ci mmol^{-1} , Amersham) was added for 2 h and the incorporation into insoluble material measured as described³³.

§ C57BL/6J *nu/nu* spleen cells activated for 48 h with $50 \mu\text{g ml}^{-1}$ LPS, partially purified from non-activated and from dead cells by velocity sedimentation³⁴ as described previously²¹. Thymidine uptake as for CTL-L cells.

¶ Influenza virus-specific helper T-cell clone vir2-15 (ref. 23), incubated at 2×10^5 cells per ml with 10 haemagglutinating units of PR8 virus and 2×10^6 2,200 rad X-irradiated BALB/c spleen cells.

¶ $50 \mu\text{g ml}^{-1}$.

* Helper T cells were the cloned, influenza virus-specific cell line vir2-15, kept on virus and syngeneic irradiated spleen cells as described²⁵, partially purified from dead cells and virus before use by repeated sedimentation over a layer of fetal calf serum. Thymidine uptake was done as with CTL-L cells (see ‡).

** Culture containing 2.5×10^6 SRBC (sheep erythrocytes) and 2×10^6 *nu/nu* C57BL/6J spleen cells, assayed at day 5 of culture as described previously^{1,19}.

†† Colony stimulating factor (CSF) activity was detected in methylcellulose culture by growth of colonies (macrophage, monocyte, granulocyte and erythrocyte colonies) from bone marrow precursor cells³⁵ as described previously³⁶.

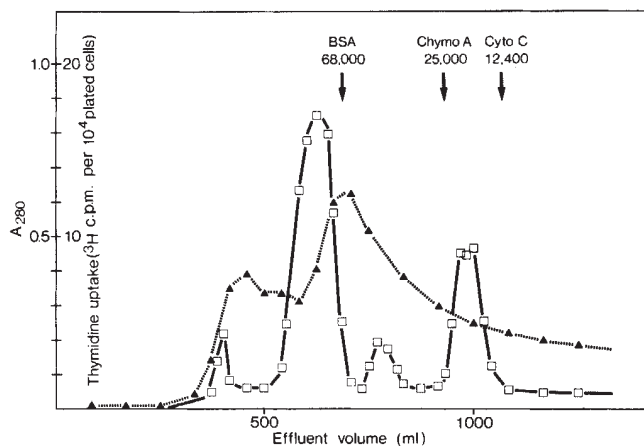


Fig. 2 Gel filtration on G100-Sephadex (column size 5 × 90 cm) of BRF produced by Con A-activated A32-26 T hybridoma cells (for details see Table 1). BRF from 1 l of conditioned medium concentrated by precipitation with ammonium sulphate (80% saturation) and dialysed against the column buffer (0.9% NaCl, 5 mM HEPES pH 7.3) was applied to the column at 4°C. The eluate collected in 10-ml fractions was monitored for absorbance at 280 nm (—□—) and for BRF activity at 3% in culture (---▲---) as described in Table 1 legend. The same column, in a parallel run, was calibrated with BSA (molecular weight 68,000), chymotrypsinogen A (25,000) and cytochrome *c* (12,400).

molecular species are structurally different, or whether they share common structural elements involved in BRF action, derived through a precursor-product relationship²⁷⁻²⁹, in a free factor/receptor-bound factor relationship^{30,31} known to exist for growth factors of other eukaryotic cells or, more trivially, by factors bound to molecules of the tissue culture medium such as bovine serum albumin.

The finding that BRF and BMF are separate activities has important bearing on our understanding of the mechanisms and the physiology of B cell regulation. Resting B cells might express BMF but not BRF receptors, while excited B cells might express both. The establishment of continuous B-cell lines and clones, analogous to functional T-cell lines and clones³² has so far been difficult. We hope that the stimulation by BRF in the absence of BMF of excited B cells not yet induced to mature will help in this experimental difficulty. Replication of B lymphocytes in the presence of BRF may continue for longer periods of time as long as the terminating action of BMF is absent.

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- Schreier, M. H., Andersson, J., Lernhardt, W. & Melchers, F. *J. exp. Med.* **151**, 194–203 (1980).
- Melchers, F., Andersson, J., Lernhardt, W., & Schreier, M. H. *Eur. J. Immun.* **10**, 679–685 (1980).
- Andersson, J. & Melchers, F. *Proc. natn. Acad. Sci. U.S.A.* **78**, 2497–2501 (1981).
- Parker, D. C., *Immun. Rev.* **52**, 115–139 (1980).
- Howard, M. et al. *J. exp. Med.* **155**, 914–923 (1982).
- Katz, D. H., Hamaoka, T., Dorf, M. E., & Benacerraf, B. *Proc. natn. Acad. Sci. U.S.A.* **70**, 2624–2628 (1973).
- Sprent, J. *Immun. Rev.* **42**, 108–148 (1978).
- Melchers, F., Andersson, J., Lernhardt, W. & Schreier, M. H. *Immun. Rev.* **52**, 89–114 (1980).
- Rosenthal, A. S. & Shevach, E. M. *J. exp. Med.* **138**, 1194–1212 (1973).
- Dutton, R. W. et al. *Prog. Immun.* **1**, 355–364 (1971).
- Schimpl, A. & Wecker, E. *Nature new Biol.* **237**, 15–17, (1972).
- Waldmann, H., Munro, A., & Hunter, P. *Eur. J. Immun.* **3**, 167–172 (1973).
- Feldmann, M., & Basten, A. *Nature new Biol.* **237**, 13–15 (1972).

- Taussig, M. J. *Nature* **248**, 234–236 (1974).
- Armerding, D., & Katz, D. H. *J. exp. Med.* **140**, 19–37.
- Delovitch, T. L., & McDevitt, H. O. *J. exp. Med.* **146**, 1019–1032 (1977).
- Schreier, M. H., & Tees, R. *Int. Archs Allergy appl. Immun.* **61**, 227–237 (1980).
- Gronowicz, E., Coutinho, A., & Melchers, F. *Eur. J. Immun.* **6**, 588–590 (1976).
- Andersson, J., Schreier, M. H., & Melchers, F. *Proc. natn. Acad. Sci. U.S.A.* **77**, 1612–1616 (1980).
- Andersson, J., Sjöberg, O., & Möller, G. *Eur. J. Immun.* **2**, 349–353 (1972).
- Andersson, J., Coutinho, A., & Melchers, F. *J. exp. Med.* **149**, 553–564 (1979).
- v. Boehmer, H., Haas, W., Köhler, G., Melchers, F. & Zeuthen, J. *Curr. Topics Microbiol. Immun.* **100**, 1–262 (1982).
- Feldmann, M. & Schreier, M. H. (eds) in *Lymphokines* Vol. 5 (Academic, New York, 1982).
- Köhler, G., Lefkowitz, I., Elliot, B., & Coutinho, A. *Eur. J. Immun.* **7**, 758–761 (1977).
- Melchers, F., Zeuthen, J., & Gerhard, W. *Curr. Topics Microbiol. Immun.* **100**, 153–163 (1982).
- Kishimoto, T. et al. *B and T cell tumors: Biological and Clinical Aspects* Vol. 24 (eds Vitetta, E. & Fox, C. F., Academic, New York, in the press.).
- Nakanishi, S. et al. *Proc. natn. Acad. Sci. U.S.A.* **73**, 4319–4323 (1976).
- Nakanishi, S. et al. *Nature* **287**, 752–755 (1980).
- Kilpatrick, D. L., Jones, B. N., Kojima, K., & Udenfriend, S. *Biochem. biophys. Res. Commun.* **103**, 698–705 (1981).
- Baker, J. B., Simmer, R. L., Glenn, K. C., & Cunningham, D. D. *Nature* **278**, 743–745 (1979).
- Linsley, P. S., Blifeld, C., Wrann, M., & Fox, C. F. *Nature* **278**, 745–748 (1979).
- Immun. Rev.* **54**, 1–266 (1981).
- Iscove, N. N. & Melchers, F. *J. exp. Med.* **147**, 923–933 (1978).
- Miller, R. G. & Phillips, R. A. *J. cell. Physiol.* **73**, 191–201 (1969).
- Schreier, M. H. & Iscove, N. N. *Nature* **287**, 228–230 (1980).
- Corbel, C. *Curr. Topics Microbiol. Immun.* **100**, 231–237 (1982).

LFA-1 but not Lyt-2 is associated with killing activity of cytotoxic T lymphocyte hybridomas

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The mechanism by which cytotoxic T lymphocytes (CTL) recognize and lyse target cells is largely unknown. Recently, two lymphocyte cell-surface glycoproteins, Lyt-2¹⁻⁴ and LFA-1 (lymphocyte function-associated antigen)⁵⁻⁸, have been implicated in the killing activity of murine CTL. This suggestion was based mainly on the unique ability of antibodies directed against Lyt-2¹⁻⁴ and LFA-1⁵⁻⁸ to inhibit cytotoxicity of CTL by affecting the killer cell. Lyt-2, a glycoprotein composed of subunits of molecular weights of 30,000–35,000 (30–35K), has been proposed as a common marker for CTL⁹, essential for specific recognition of target cells¹⁰. This view, however, is incompatible with other results¹¹⁻¹⁴. The newly detected LFA-1 molecule, containing subunits of 95K and 180K^{5-8,15}, is associated with the ability of CTL to bind target cells^{16,17}. Most analyses of CTL structure-function relationships are ambiguous since they have been obtained with heterogeneous CTL populations. We have recently developed CTL-hybridomas which lyse specifically allogeneic tumour cells *in vitro*¹⁸ and grow independently of exogenous stimuli¹⁹. In this study we have tested the relevance of Lyt-2 and LFA-1 antigens to cytotoxicity by analysing the CTL-hybridomas with a panel of anti-LFA-1 and anti-Lyt-2 monoclonal antibodies. We show that two monoclonal CTL-hybridomas have the phenotype LFA-1⁺ and Lyt-2⁻ and that their killing activity is inhibited by anti-LFA-1 but not by anti-Lyt-2. The results suggest that LFA-1 is involved in CTL-cytotoxicity, while Lyt-2 molecules are not required.

CTL somatic hybrids, in which elimination of functionally irrelevant molecules could occur due to chromosomal segregation or to repression controlled by the tumour cell parent, provide a new homogeneous and antigen-specific source for

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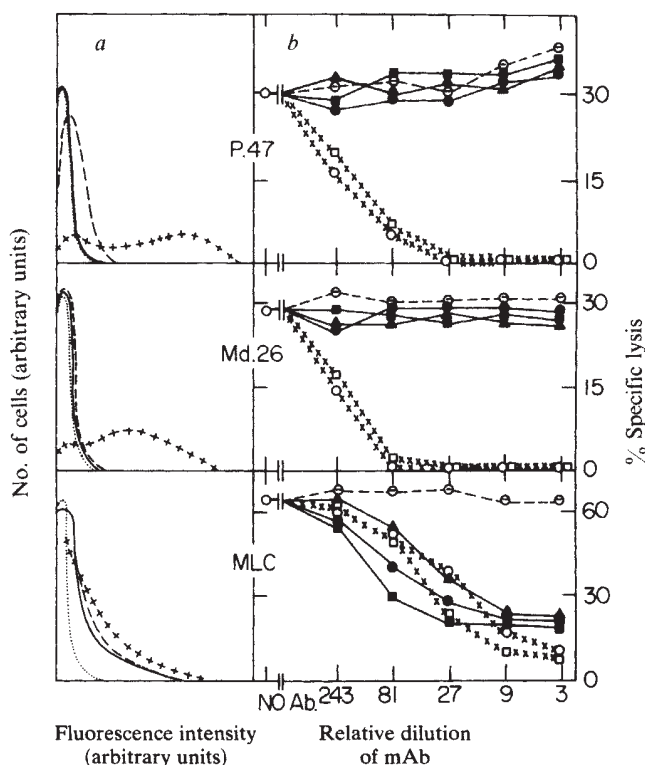


Fig. 1 Expression of Lyt and LFA-1 antigens on CTL-hybridomas and the effect of anti-Lyt and anti-LFA-1 mAb on cytotoxicity. *a*, Cytofluorometric analysis of Lyt-1, Lyt-2 and LFA-1 on CTL and CTL-hybridomas was done by reacting 2×10^6 MLC effector cells (primary BALB/c anti-C57BL/6 spleen cells stimulated for 5 days at 1:1 ratio) or CTL-hybridoma clones P.47 or Md.26 with the following monoclonal antibodies: anti-Lyt-1 (---○) and anti-Lyt-2 (—○) produced by hybridomas 53.7.3 and 53.6.7, respectively²² (these were obtained from the Cell Distribution Center of the Salk Institute, San Diego); anti-LFA-1 (+++) produced by hybridoma H35-89.9⁷ and concentrated from ascites by 50% $(\text{NH}_4)_2\text{SO}_4$. Washed cells were stained with fluoresceinated rabbit anti-rat immunoglobulin. Samples were analysed using a fluorescence-activated cell sorter (FACS II). For each cell preparation, a control staining with the fluoresceinated reagent alone (····) is shown. Data are expressed in arbitrary units as obtained from the cell sorter. *b*, The effect of anti-Lyt and anti-LFA-1 monoclonal antibodies on cytotoxicity of MLC and CTL hybridomas was studied by incubating 10^4 effector cells in microtitre plates with different dilutions of the following monoclonal antibodies: anti-Lyt-1, 53.7.3²² five times concentrated (---○); anti-Lyt-2 were 53.6.7²² five times concentrated (▲); H35-17.2¹⁷ 1:3 diluted (●); and M12/7.2⁵ 1:10 diluted (■); anti-LFA-1 were H35-89.9⁷ at 1:1,000 dilution (+++○); and M17/5.2⁵ at 1:30 dilution (+++□). The concentrations of antibodies listed above indicate the original stocks which were serially diluted in the assay. After 20 min incubation at 37°C in 100 μl reaction medium (RPMI containing 10% fetal calf serum and 10 mM HEPES pH 7.4), 10^4 ^{51}Cr -labelled, neuraminidase-treated¹⁸ EL4 target cells were added in 50 μl reaction medium. Plates were spun for 5 min at 500 r.p.m. and incubated for 5 h at 37°C. Lysis was stopped by cooling, plates were spun for 10 min at 2,000 r.p.m. and 75 μl supernatants were taken to determine the ^{51}Cr release. Spontaneous release was 12%.

studies of specific effector structures participating in cytotoxicity. We analysed the effect of various monoclonal antibodies on two unrelated CTL-hybrid subclones, Md. 26 and P. 47, that had been derived from BALB/c anti-EL4 (H-2^d anti-H-2^b) CTL following secondary stimulation either in mixed lymphocyte culture (MLC) or *in vivo* (peritoneal exudate lymphocytes, PEL), respectively¹⁸. These monoclonal CTL-hybridomas retained their killing specificity towards H-2^b tumour target cells during prolonged continuous growth in

culture²⁰ and exhibited this capacity while the studies described here were carried out. The monoclonal antibodies examined had been raised against CTL and selected according to their ability to inhibit killing by binding to the effector cells^{5,7,17,21}.

The presence of the differentiation antigens Lyt-1, Lyt-2 and LFA-1 on the CTL hybridomas and heterogeneous MLC population was examined by immunofluorescence staining. The analysis (Fig. 1*a*) demonstrates the presence of all three antigens on MLC, low levels of Lyt-1 on P but not on M hybridomas and high density of LFA-1 on both hybridomas. None of the CTL-hybridomas were stained with anti-Lyt-2. Additional monoclonal anti-Lyt-2 (H35-17.2¹⁷, M12/4, M12/5 and M12/7.2²¹), failed to stain the hybridomas, while several anti-LFA-1 monoclonal antibodies (H35-89.9⁷ and M17.5.2⁵) stained over 85% of the hybrid cells (not shown). The relevance of Lyt-2 and LFA-1 to the killing activity was further studied by testing the effect of the monoclonal antibodies on cytotoxicity (Fig. 1*b*). The hybridomas or BALB/c anti-C57BL/6 MLC were incubated with ^{51}Cr -labelled EL4 target cells in the presence of diluted antibody and specific killing was determined. As previously reported, cytotoxicity of MLC was inhibited by anti-Lyt-2¹⁻⁴ and by anti-LFA-1⁵⁻⁸ and was unaffected by anti-Lyt-1. The killing activity of both CTL-hybridomas was likewise unaffected by anti-Lyt-1 and completely abolished by two anti-LFA-1 antibodies. However, unlike parental MLC, the cytotoxicity of the hybridomas was unaffected by three different anti-Lyt-2 monoclonal antibodies. The absence of detectable Lyt-2 on the surface of the CTL-hybridoma was further confirmed by preincubation of both M and P hybrid cells with two different anti-Lyt-2 antibodies in the presence of complement (Fig. 2). Such pretreatment completely eliminated the cytotoxicity of parental MLC but did not affect the cytolytic potential of hybridomas. The inability to detect Lyt-2 on the

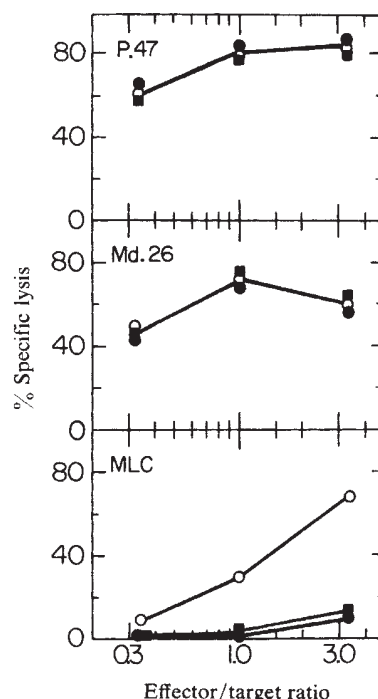


Fig. 2 The effect of pretreatments with anti-Lyt-2 and complement on the cytotoxicity of CTL-hybridomas and MLC. CTL-hybridomas and primary MLC (see Fig. 1 legend) were preincubated for 45 min at 37°C with anti-Lyt-2; H35-17.2¹⁷ (●) or M12/7.2⁵ (■) and 1:10 dilution of fresh guinea pig serum as complement or with complement alone (○). After washing, treated cells were diluted and cytotoxicity was tested on 10^4 neuraminidase-treated ^{51}Cr -labelled EL4 target cells. E/T ratio was calculated according to number of effector cells before treatment. Incubation was for 5.5 h and spontaneous release was 18%.

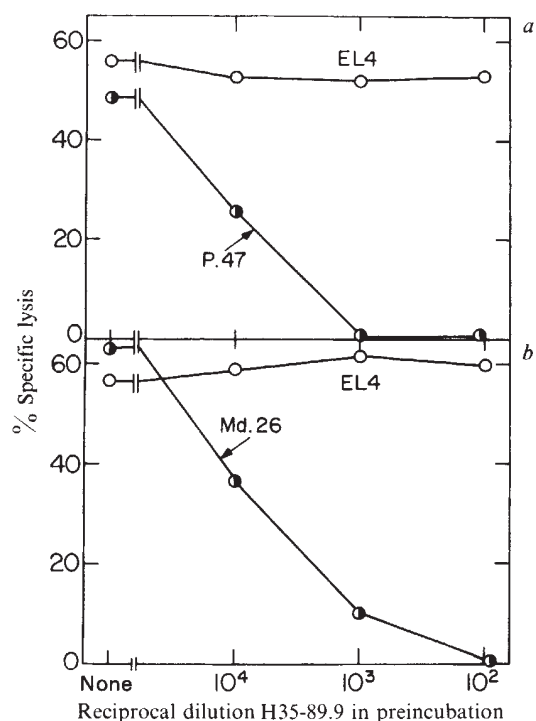


Fig. 3 Preincubation of CTL-hybridomas but not of EL4 target cells with monoclonal anti-LFA-1 inhibits cytotoxicity. Isolated CTL-hybridomas or neuraminidase-treated ⁵¹Cr-labelled EL4 cells were incubated with anti-LFA-1 H35-89.9⁷ at the indicated dilutions for 60 min at 37 °C. Cells were washed three times from excess of monoclonal antibody and cytotoxic reactions were completed with an equivalent number of the missing untreated effector or target cells. Killing assays contained 10⁴ CTL-hybridomas (either P.47 (a) or Md.26 (b)).

CTL hybridomas did not result from a wrong specificity or a low titre of the antibody used; since antibody 53.6, which recognizes a nonpolymorphic portion of Lyt-2 molecule²² and two other monoclonal anti-Lyt-2 molecules, did bind to parental MLC killers and inhibited very effectively their cytotoxicity, while the same monoclonal antibodies could neither bind to the hybrid cells (Figs 1a and 2) nor affect their killing activity (Fig. 1b). Furthermore, more than a single Lyt-2 antigenic determinant was absent, since the hybridomas were unreactive with antibodies M12/4, M12/5 and M12/7, which define three topographically distinct Lyt-2 determinants (not shown).

In contrast to anti-Lyt-2, four different monoclonal anti-LFA-1 molecules were all equally effective in inhibiting specific killing of EL4 and ALB.B2 target cells by the hybridomas (Fig. 1b and not shown). Since LFA-1 molecules are expressed not only on the CTL-hybridomas but also on EL4 (ref. 15 and our unpublished results), which had been used as target cells in these experiments, it was necessary to examine whether anti-LFA-1 exerts its inhibitory effect on killing through its binding to the target cells or to the effector cells, as previously suggested⁵⁻⁸. This was tested by preincubating anti-LFA-1 separately with either isolated CTL-hybridomas or EL4 cells and combining treated and untreated components for the killing assay after excess of the antibody was removed. Cytotoxicity of preincubated CTL hybridomas was completely abolished by bound anti-LFA-1 molecules (Fig. 3), while pretreated EL4 target cells were unaffected, showing that indeed, anti-LFA-1 inhibited killing by affecting primarily the CTL-hybridomas rather than EL4 target cells.

The hypothesis that Lyt-2 is associated with the CTL receptor was based on a genetic linkage between Lyt-2-3 locus and the V_k locus²³, on the observation that anti-Lyt-2 inhibits killing at the recognition-adhesion stage²⁴, and on a mutagenized CTL Lyt-2⁺ clone which lost in parallel Lyt-2 and the ability to kill

specifically TC¹⁰. Recently, however, MacDonald *et al.*¹⁴ reported that some CTL which express Lyt-2 are not inhibited by anti-Lyt-2. In addition, it becomes apparent that CTL populations¹² and long-term lines^{11,13} directed against Ia antigens (class II) major histocompatibility complex (MHC) are Lyt-1⁺2⁺ and helper cells specific for class I MHC antigens are Lyt-1⁺2⁺ and their helper function is inhibited by anti-Lyt-2²⁶. These results prompted Swain²⁶ to suggest that Lyt phenotype correlates primarily with the class of MHC antigen recognized rather than with function (see also ref. 27 for a recent discussion). Our observation that functional CTL-hybridomas are Lyt-2 negative support the notion that Lyt-2 molecules are not required for specific cytotoxicity. The H-2 specificity of these CTL-hybridomas was recently mapped to the D region of H-2^b by using target cells from recombinant mice²⁸ and by inhibition of the hybridoma-mediated target cell killing with anti-H-2D^b¹⁹. In this respect, the CTL-hybridomas seem to be exceptional, since all CTL directed against class I MHC were reported so far as Lyt-2⁺. However, hybridomas are often subjected to selective loss of some parental chromosomes or to repression of specific genes, hence it is possible that the CTL hybridomas analysed here originated from Lyt-2⁺ parental CTL whose Lyt-2 expression was lost. Regardless of the history of these cells, the mere fact that they specifically lyse target cells without expressing Lyt-2 antigens strongly suggests that Lyt-2 molecules are not essential for either target cell recognition or binding. This is in contrast to previous suggestions that Lyt-2 is an antigen receptor. However, it is possible that Lyt-2 is a non-antigen-specific receptor that, together with the antigen receptor, contributes to the avidity of the CTL for the target cells^{17,21}. In cases where the antigen receptor has low avidity, loss of the Lyt-2 interaction would result in loss of CTL activity, explaining the antibody inhibition¹⁻⁴ and Lyt-2 mutant results¹⁰. Thus, while in some cases Lyt-2 may contribute to CTL activity, we conclude that it is not essential and is distinct from the antigen receptor.

LFA-1 molecules, unlike Lyt-2, may be an essential structure of functional CTL. This conclusion is based on the observation that *all* CTL populations and clones examined so far express LFA-1 and their cytotoxicity is inhibited by anti-LFA-1⁵⁻⁸. The formation of effector target cell conjugates is inhibited by anti-LFA-1^{16,17}, indicating that the binding stage is affected. The ability of anti-LFA-1 to inhibit specific lysis irrespective of the TC specificity^{7,16}, as well as concanavalin A mediated nonspecific killing^{7,17}, emphasizes the general role of LFA-1 in a fruitful CTL target cell interaction. Previous findings that anti-LFA-1 interfere with other T-cell functions like proliferative MLC responses, macrophage-dependent antigen-specific proliferation, and T cell-dependent antibody responses to red cells^{7,16,21}, suggest that LFA-1 is crucial for the interaction of T cells with other cells. The fact that a panel of anti-LFA-1 monoclonal antibodies inhibited very efficiently the specific cytotoxicity of CTL-hybridomas (Figs 1b, 3) and immunoprecipitated from the hybrid cells the 95K and 180K LFA-1 subunits (not shown) indicates that the same LFA-1 molecules that participate in parental CTL cytotoxicity also participate in the specific target cell killing induced by the hybridomas. The monoclonal CTL hybridomas which grow rapidly in culture and retain specific killing activity for months without added stimuli, provide an unlimited and homogeneous source for studying the molecular basis for the role of LFA-1 in T cell-mediated cytotoxicity.

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1. Nakayama, E., Shiku, H., Stockert, E., Oettingen, H. F. & Old, L. J. *Proc. natn. Acad. Sci. U.S.A.* **76**, 1977-1981 (1979).
2. Shinohara, N. & Sachs, D. H. *J. exp. Med.* **150**, 432-444 (1979).
3. Hollander, N., Pillemer, E. & Weissman, I. *J. exp. Med.* **152**, 674-687 (1980).

4. Sarmiento, A., Glasebrook, A. L. & Fitch, F. W. *J. Immun.* **125**, 2665-2672 (1980).
5. Davignon, D., Martz, E., Reynolds, T., Kürzinger, K. & Springer, T. A. *Proc. natn. Acad. Sci. U.S.A.* **78**, 4535-4539 (1981).
6. Kürzinger, K., Reynolds, T., Germann, R. N., Davignon, D., Martz, E. & Springer, T. A. *J. Immun.* **127**, 596-602 (1981).
7. Pierres, M., Goridis, C. & Golstein, P. *Eur. J. Immun.* **12**, 60-69 (1982).
8. Dialynas, D., Loken, M., Sarmiento, M. & Fitch, F. W. *Mechanisms of Cell-Mediated Cytotoxicity* (eds Clark, W. R. & Golstein, P.) 547-556 (Plenum, New York, 1982).
9. Cantor, H. & Boyse, E. A. *Cold Spring Harb. Symp. quant. Biol.* **41**, 23-31 (1977).
10. Dialynas, D. P., Loken, M. R., Glasebrook, A. L. & Fitch, F. W. *J. exp. Med.* **153**, 595-604 (1981).
11. Swain, S. L., Dennert, G., Wormsley, S. & Dutton, R. *Eur. J. Immun.* **11**, 175-180 (1981).
12. Vidovic, D., Juretic, A., Nagy, Z. A. & Klein, J. *Eur. J. Immun.* **11**, 499-504 (1981).
13. Pierres, A., Schmitt-Verhulst, A.-M., Buferne, M., Golstein, P. & Pierres, M. *Scand. J. Immun.* (in the press).
14. MacDonald, H. R., Thiernes, N. & Cerottini, J. C. *J. Immun.* **126**, 1671-1675 (1981).
15. Kürzinger, K., Ho, M. K. & Springer, T. A. *Nature* **296**, 668-670 (1982).
16. Davignon, D., Martz, E., Reynolds, T., Kürzinger, K. & Springer, T. A. *J. Immun.* **127**, 590-595 (1981).
17. Golstein, P. *et al. Immun. Rev.* (in the press).
18. Kaufmann, Y., Berke, G. & Eshhar, Z. *Proc. natn. Acad. Sci. U.S.A.* **78**, 2502-2506 (1981).
19. Eshhar, Z., Waks, T., Oren, T., Berke, G. & Kaufmann, Y. *Curr. Topics Microbiol. Immun.* **100**, 11-17 (1982).
20. Kaufmann, Y., Berke, G. & Eshhar, Z. *Lymphokines, Monoclonal, T Cells and Their Products* Vol. 5 (eds Feldman, M. & Schreier, M. H.) 277-289 (Academic, New York, 1982).
21. Springer, T. A., Davignon, D., Ho, M.-K., Kürzinger, K., Martz, E. & Sanchez-Madrid, F. *Immun. Rev.* **68** (in the press).
22. Ledbetter, J. A. & Herzenberg, L. A. *Immun. Rev.* **47**, 63-89 (1979).
23. Gibson, D. M., Taylor, B. A. & Cherry, M. J. *Immun.* **121**, 1585-1590 (1978).
24. Fan, J., Ahmed, A. & Bonavida, B. *J. Immun.* **125**, 2444-2453 (1980).
25. Swain, S. L. & Fanfili, P. R. *J. Immun.* **122**, 383-391 (1979).
26. Swain, S. L. *Proc. natn. Acad. Sci. U.S.A.* **78**, 7101-7105 (1981).
27. Simpson, E. *Nature* **295**, 366-367 (1982).
28. Kaufmann, Y. *Transplant Proc.* (in the press).

Restricted expression of viral glycoprotein in cells of persistently infected mice

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For a virus to cause persistent infection in a host, a unique host-virus relationship is required. The virus *per se*, or variant(s) arising *in vivo*, must be noncytopathic for the host cell that it infects. Furthermore, the infected cell must escape recognition by the host's immune system, which eliminates virus and virus-infected cells. Several models of persistent infection *in vitro* demonstrate selective loss of viral gene products normally represented at the cell surface as opposed to viral products found inside the cell¹⁻⁵. Decreased glycoprotein (gp) expression on the surface of an infected cell correlates directly with the ability of that cell to resist killing by both cytotoxic lymphocytes and cytotoxic antibody and complement^{1,3,6}. The question remains of whether a corresponding selective loss of viral glycoprotein expression occurs in persistently infected cells *in vivo*. We have used monoclonal antibodies as probes to define the viral glycoprotein and nucleoprotein (NP) molecules expressed in individual cells from several tissues of mice persistently infected with lymphocytic choriomeningitis virus (LCMV). We report here a selective decrease in the expression of viral glycoprotein in single cells, during persistent infection *in vivo*.

LCMV is noncytopathic and easily induces persistent infection in its natural host, the mouse (for review see refs 7, 8). The virus contains a negative-stranded two-segmented RNA genome and three structural polypeptides. Of these, two are glycosylated (gp1 and gp2), and one is the nonglycosylated NP; the molecular weights are 4.3×10^4 , 3.6×10^4 and 6.3×10^4 for gp1, gp2 and NP, respectively⁷. Collectively, the glycoproteins make up ~30% of the virion mass. The gp1 and gp2 form in infected cells in equimolar amounts by proteolytic cleavage from a common precursor, gpC, and are distinct according to tryptic peptide mapping⁹; gp1 is the predominant viral polypeptide accessible to radiolabelling or antibody binding on the

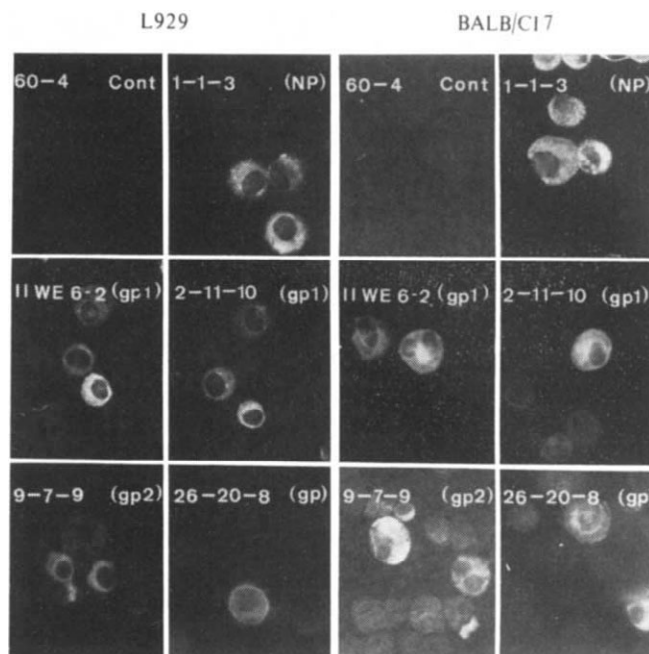


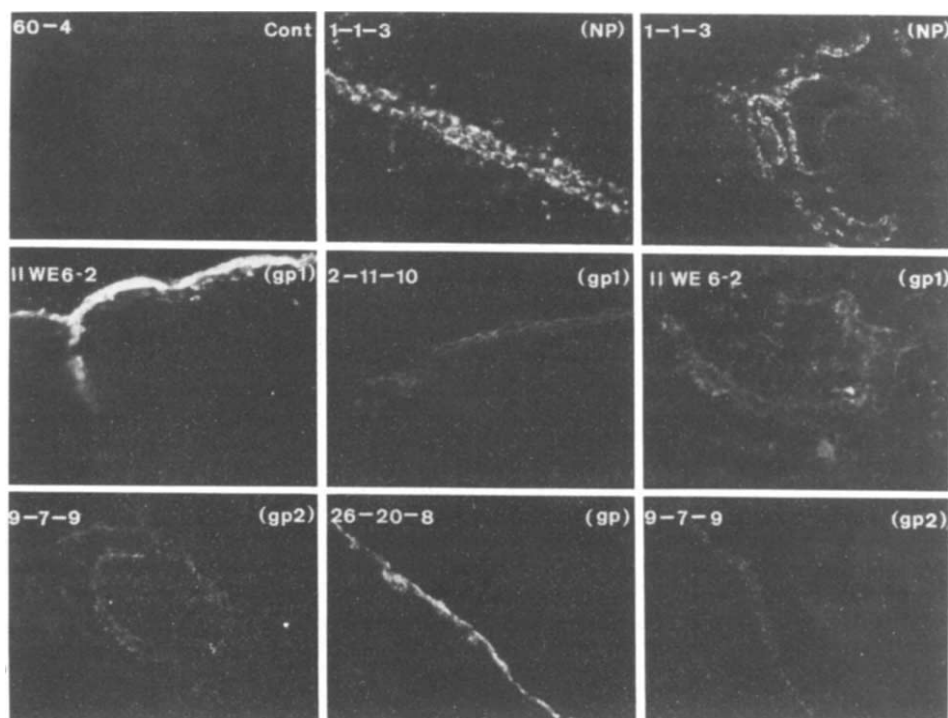
Fig. 1 Expression of viral glycoprotein and NP by cultured cells during acute infection with LCMV, Armstrong 1371 strain. L929 or BALB/C17 cells were infected with LCMV at a multiplicity of infection (MOI) of 3 and studied 2 days later for the expression of viral gp1, gp2 or NP. Cells were fixed with ether/alcohol (1:1) for 20 min, 95% ethanol for 10 min, then washed in buffer and stained with the monoclonal antibodies shown. 60-4 is a monoclonal antibody to the glycoprotein (G) protein of vesicular stomatitis virus and served as a negative control. After 30 min incubation, the cells were washed twice in buffer and stained with monospecific goat anti-mouse immunoglobulin antibody conjugated to rhodamine. After 20 min, the cells were triple-washed in buffer, covered with glycerol-Tris buffer and a coverslip and studied by fluorescence microscopy.

surfaces of infected cells or virions⁹. A large battery of monoclonal antibodies against these three structural LCMV polypeptides was obtained by using a modification of the Kohler-Milstein method¹⁰. Specificities of the various monoclonal antibodies were then determined after immune precipitation of ³⁵S-labelled LCMV polypeptides, as described elsewhere¹⁰. The antibodies were used as molecular probes to analyse the expression of gp1, gp2 and NP by indirect immunofluorescence assay^{6,10}.

We first determined the expression of viral gene products during acute LCMV infection of cultured cells and subsequently of intact, adult animals. Viral gp1, gp2 and NP were evident in the cytoplasm of both L929 and BALB/c clone (Cl) 7 cells 2 days after initiating infection (see Fig. 1). Similar results were obtained when either hybridoma cells or N115 neuroblastoma cells were similarly infected and analysed for each viral polypeptide, or when individual cells were stained with two fluorochrome dyes¹¹ (one dye was used to detect the NP, the other to detect viral glycoproteins). For these and other studies, the antiviral gp1 and gp2 monoclonal antibodies were pooled and used as a reference glycoprotein reagent. The plasma membrane showed expression of molecules of viral glycoprotein only on the surfaces of acutely infected cells. In subsequent tests, ependyma, choroid and meningeal cells from several individual adult mice of the C3H/St, BALB/WEHI and SWR/J strains acutely infected with LCMV (60 plaque-forming units (PFU), i.e.) expressed both types of viral glycoprotein and NP when sampled 5 days after infection (Fig. 2).

In contrast to our findings in acutely infected mice and cells, mice persistently infected *in vivo*, as well as cultured mouse cells persistently infected *in vitro* with LCMV, selectively lost viral gp expression with time but retained viral NP. For example,

Fig. 2 Expression of viral glycoprotein and NP during acute infection of adult C3H/St mice with 60 PFU of LCMV, Armstrong 1371 strain. Expression of both viral glycoprotein and NP is evident in cells of the ependyma (centre—upper row), choroid plexus (right—upper row; right—middle row; left—lower row) and meninges (left and centre—middle row; centre and right—lower row). Brains collected 5 days after infection were snap-frozen with liquid nitrogen, cut into 4- μ m sections in a cryostat and mounted on glass slides. Slides containing the brain sections were fixed and stained as described in Fig. 1 legend. Similar results were seen when anti-gp1 and -gp2 monoclonal antibodies were pooled and then used as a staining reagent. Similar results were observed with SWR/J and BALB/W mice.



at 60 days after the initiating infection, and for as long as 360 days after the experiment ended, persistently infected cells expressed no detectable viral gp on their surfaces and only rarely was it found in the cytoplasm. This observation has been confirmed in studies of single cells from animals infected *in vivo* (Fig. 3 and Table 1). For the *in vivo* study, persistent infection was established by inoculating newborn SWR/J, BALB/WEHI or C3H/St mice i.c. with 60 PFU of LCMV, Armstrong 1371 strain¹². Such neonatally infected mice carry virus in their tissues throughout life despite mounting an antiviral immune response^{7,12}. We evaluated cells from the brain, liver, kidney and pituitary tissues of such mice at 2, 5, 15, 30, 60, 84 and 180 days after initiating infection, for expression of viral glycoprotein or NP, using monoclonal antibodies (Table 1). At the second and fifth days after infection, neurones of the central nervous system (CNS), parenchymal cells of the liver, tubular epithelial cells of the kidney and cells in the anterior

lobe of the pituitary expressed viral glycoprotein and NP in a manner similar to acutely infected cells from adult mice (Fig. 2) and acutely infected cells in culture (Fig. 1). By 15 days after infection, most but not all infected cells demonstrated both glycoprotein and NP polypeptides. However, at 30 days and later, individual infected cells located in the CNS, liver, kidney and anterior lobe of the pituitary had lost viral glycoprotein but retained the expression of viral NP (Fig. 3, Table 1).

These studies suggest that the synthesis of viral glycoprotein decreases in cells during persistent infection *in vivo*, resulting in a decrease in its expression on the surfaces of the cells. Decreased viral glycoprotein expression possibly allows persistently infected cells to resist assault by virus-specific immune reactants. By this means, a noncytopathic virus may persist throughout the lifespan of an infected cell and the cell could remain alive, unaffected by the host immune surveillance mechanism.

Although the cellular content of viral glycoprotein diminished, that of viral NP did not. Despite the accumulation of viral NP in specialized cells of the host, no morphological alteration was evident by either light or electron microscopy^{13,14}. Further, electron microscopic study of infected neuronal cells from persistently infected mice reacted with monoclonal antibodies confirmed that NP accumulated inside the cell, usually aggregated in the area of polyribosomes¹⁴, while viral gp inside the cell was reduced and the virus became restricted from budding at the cell surface.

These findings in individual neuronal, hepatic, epithelial and endocrine cells from mice persistently infected with LCMV *in vivo* extend the results seen *in vitro*^{1-5,15,16}. Such persistently infected cells develop a quantitative 10- to 50-fold decrease in viral glycoprotein synthesis and glycoprotein expression compared to NP synthesis and expression in acutely infected cells^{1,15}. Our *in vitro* studies suggest that the decrease in glycoprotein expression parallels the generation of defective interfering virus (DIV)^{1,15}. Whether DIV is generated *in vivo* during natural or experimentally induced LCMV infection is unknown, but is now being studied.

Persistent LCMV infection of cultured differentiated neuroblastoma cells correlates directly with a significant dysfunction in the synthesis or degradation of acetylcholine due to the reduction in acetylcholine transferase or esterase, respectively¹⁶. Similar alterations in differentiated functions have

Table 1 Dissociated expression of LCMV glycoprotein and NP during persistent virus infection

		Expression of viral gene products in neurones							
		Acute infection		Persistent infection					
		Day 3-5		Day 5		Day 15		Day >80	
Strain		gp	NP	gp	NP	gp	NP	gp	NP
C3H/St	Adult	8/8*	8/8	—†	—	—	—	—	—
BALB/W	Adult	5/5	5/5	—	—	—	—	—	—
SWR/J	Adult	8/8	8/8	—	—	—	—	—	—
C3H/St	Newborn	—	—	5/5*	5/5	9/15	15/15	1/8	8/8
BALB/W	Newborn	—	—	5/5	5/5	ND	ND	1/20	20/20
SWR/J	Newborn	—	—	5/5	5/5	ND	ND	3/25	25/25

Individual neurones were studied for the expression of viral glycoprotein (gp) or NP by using appropriate monoclonal antibody and antibody to mouse immunoglobulin conjugated to rhodamine or fluorescein isothiocyanate. Sections (4 μ m) through the anterior one-third of the temporal lobe were fixed in ether/alcohol (1:1) and 95% alcohol before staining as described elsewhere¹². At least 100 individual neurones per section were studied. Acute infection was induced in 4-5-week-old mice by i.c. inoculation of 60 PFU of LCMV, Armstrong strain 1371; persistent infection followed inoculation of newborn mice with a similar route of inoculation and dose of virus. Infected mice were killed after infection, on the days indicated. ND, not determined.

* Number of mice studied that contained one or more cells expressing a specific viral polypeptide/total number of mice.

† The virus dose used in adult mice for acute infection leads to death by day 6-8.

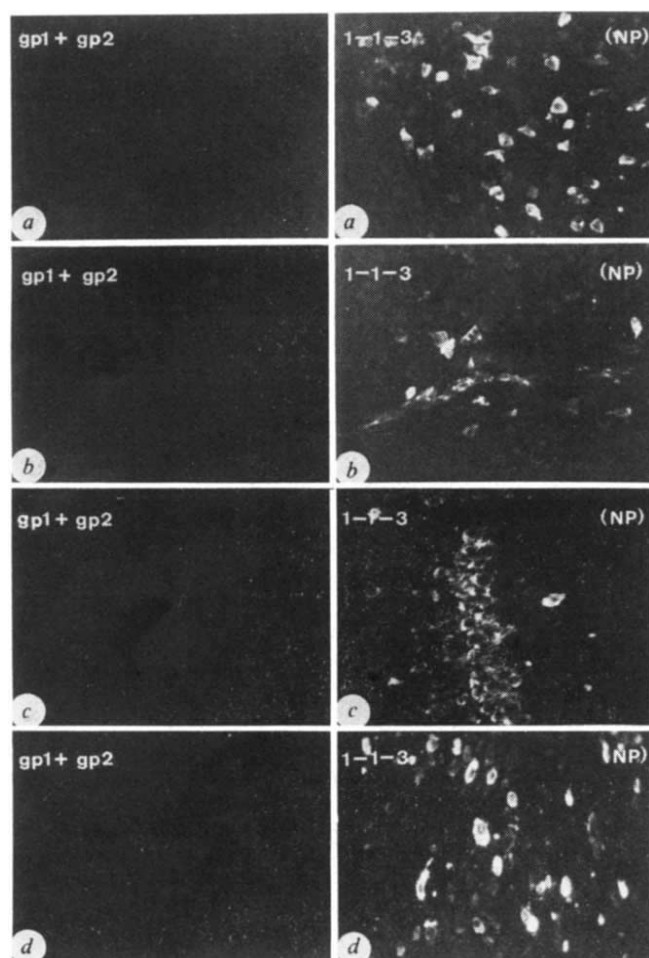


Fig. 3 Lack of viral glycoprotein but presence of NP in individual neuronal cells from C3H/St mice persistently infected with LCMV. Newborn mice were inoculated with 60 PFU of LCMV, Armstrong 1371 strain and killed 84 days later. Neuronal cells of four mice (a-d) expressed NP, but in the adjunct 4- μ m section no glycoprotein is apparent for the same cell. The collection, sectioning and staining of the tissues was the same as described in Fig. 2 legend, except that antibodies to gp1 and gp2 from hybridomas were pooled and then used as the staining reagent. Similar results were observed for SWR/J and BALB/W mice.

recently been observed after viral infection *in vitro* when hybridoma cells no longer produced monoclonal antibodies, and *in vivo* when differentiated growth hormone-producing cells made significantly less growth hormone^{3,17}. Hence, by avoiding immune responses directed against the cells they infect, noncytopathic viruses may significantly alter physiological homeostasis by causing a dysfunction in the cells of the endocrine, immune or other differentiated systems; the end result is a new mechanism for the production of virus-induced diseases. Whether these biochemical dysfunctions at the cellular level are directly related to the accumulation of viral products in a manner akin to a storage disease is a matter for further study. The results reported here for persistent LCMV infection resemble the overall pattern observed during persistent measles virus infection (subacute sclerosing panencephalitis). Neuronal cells in biopsies from these patients showed a decrease in or lack of measles virus glycoprotein but they accumulated measles virus nucleocapsids internally¹⁸. Thus, other persistent infections may produce similar findings *in vivo*.

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1. Welsh, R. M. & Oldstone, M. B. A. *J. exp. Med.* **145**, 1449-1468 (1977).
2. Joseph, B. S., Perrin, L. H. & Oldstone, M. B. A. *J. gen. Virol.* **30**, 329-337 (1976).
3. Oldstone, M. B. A. & Fujinami, R. S. in *Virus Persistence, Symp. 33 Soc. gen. Microbiol.* (eds Mahy, B. W. J., Minson, A. C. & Darby, G. K.) 185-202 (Cambridge University Press, 1982).
4. Holland, J. J. *et al.* *J. gen. Virol.* **33**, 193-211 (1976).
5. Gimenez, H. B. & Compans, R. W. *Virology* **107**, 229-239 (1980).
6. Oldstone, M. B. A. & Dixon, F. J. *J. Immun.* **107**, 1274-1280 (1971).
7. Buchmeier, M. J., Welsh, R. M., Dutko, F. J. & Oldstone, M. B. A. *Adv. Immun.* **30**, 275-331 (1980).
8. Lehmann-Grube, F. *Virology Monogr.* Vol. 10 (Springer, Vienna, 1971).
9. Buchmeier, M. J. & Oldstone, M. B. A. *Virology* **99**, 111-120 (1979).
10. Buchmeier, M. J., Lewicki, H. A., Tomori, O. & Oldstone, M. B. A. *Virology* **113**, 73-85 (1981).
11. Yefenof, E., Klein, G., Jondal, M. & Oldstone, M. B. A. *Int. J. Cancer* **17**, 693-700 (1976).
12. Oldstone, M. B. A. & Dixon, F. J. *J. exp. Med.* **129**, 483-505 (1969).
13. Oldstone, M. B. A. & Dixon, F. J. *Fedn Proc.* **33**, 2057-2059 (1974).
14. Rodriguez, M., Buchmeier, M., Lampert, P. & Oldstone, M. *Am. J. Path.* (in the press).
15. Welsh, R. M. Jr & Buchmeier, M. J. *Virology* **96**, 503-515 (1979).
16. Oldstone, M. B. A., Holmstoen, J. & Welsh, R. M. Jr *J. cell. Physiol.* **91**, 459-472 (1977).
17. Oldstone, M. B. A. *et al. Science* (in the press).
18. Iwasaki, Y. & Koprowski, H. *Lab. Invest.* **31**, 187-196 (1974).

DNA strand breaks and ADP-ribosyl transferase activation during cell differentiation

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The nuclear enzyme ADP-ribosyl transferase (ADPRT) catalyses the formation of poly(ADP-ribose)-modified chromatin proteins from NAD⁺ (refs 1-5) and is entirely dependent on DNA⁶ containing nicks⁷⁻¹¹. Nuclear ADPRT activity is required for efficient DNA excision repair^{12,13}, probably because it regulates DNA ligase activity¹⁴. Indirect evidence has suggested that ADPRT activity may also be involved in control of gene expression and cell differentiation¹⁵⁻²¹. We report here an obligatory involvement of ADPRT activity in the differentiation of muscle cells. Inhibitors of ADPRT activity reversibly inhibit both fusion of myoblasts to form multi-nucleate muscle fibres and the differentiation-specific increase in creatine phosphokinase (CPK) activity. These two markers of differentiation can also be reversibly inhibited by depriving the cells of nicotinamide and thus lowering their cellular NAD content. Specific gene expression sometimes requires gene rearrangements or DNA transposition; this implies that DNA strand-breaking and rejoining might be involved in gene expression. We also describe the appearance during cytodifferentiation of single-strand DNA breaks which are not due to a general deficiency in DNA repair.

Primary chick myoblasts in culture undergo an initial phase of proliferation before terminal differentiation which is marked by the fusion of myoblast cell membranes to form multinucleate syncytia of muscle fibres, the cessation of DNA biosynthesis and cell division, and a substantial increase in CPK activity, largely due to *de novo* synthesis of a muscle-specific isozyme of this protein (Fig. 1).

ADPRT activity can be estimated by the rate of incorporation of radioactivity from ³H-adenine-NAD⁺ into acid-insoluble material in permeabilized cells. Two forms of ADPRT activity may be measured¹⁰; the intrinsic, physiological, endogenous activity in the absence of any experimentally induced DNA

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strand breaks, and the total, potential enzyme activity displayed by deoxyribonuclease to generate the optimal number of breaks.

During the initial, proliferative phase of culture there is a low, constant physiological ADPRT activity (Fig. 1). Just before

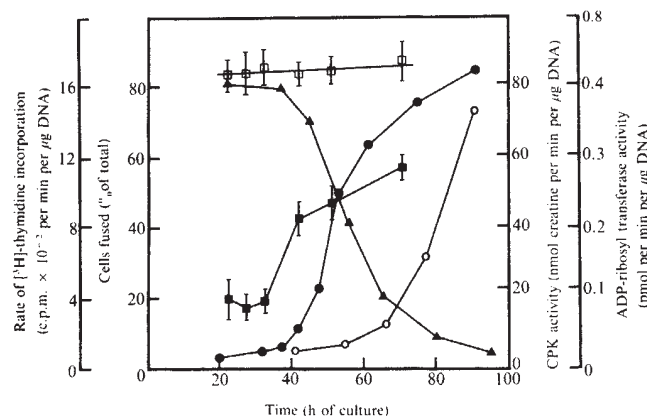


Fig. 1 Muscle cell differentiation *in vitro*. Myoblasts were isolated from the thigh muscle of 12-day-old chick embryos by mechanical dissociation and selective plating according to the method of Mån *et al.*³¹. Cell suspensions were seeded at 0.5×10^6 cells per 6-cm collagen-coated plates in 2.5 ml M199 medium (Earle's salts) supplemented with 10% (v/v) selected horse serum, 2.5% (v/v) embryo extract, 1 mM glutamine, 50 U/ml⁻¹ penicillin and 50 $\mu\text{g ml}^{-1}$ streptomycin. Cultures were then incubated, at 37 °C, in 95% air 5% CO₂ and subjected to a complete change of medium at 24 h. The specific substrate of ADPRT is NAD⁺, to which intact cell membranes are impermeable. Isolated nuclei are unsuitable because the process of nuclear isolation induces DNA strand breaks and activates ADPRT^{9,10}; thus it is necessary to use permeabilized cells to measure ADPRT activity. The myoblast and myotubes were made permeable to ³H-NAD by a mild, brief hypotonic treatment by a modification of the method of Reinhard *et al.*³². Muscle cells on 6-cm plates were washed three times with ice-cold Pucks saline A (5.4 mM MgCl₂, 137 mM NaCl, 4.2 mM NaHCO₃ and 5.5 mM glucose). The plates were then flooded with 1.0 ml of a hypotonic solution containing 9 mM HEPES pH 7.8, 5 mM dithiothreitol, 4.5% (w/v) dextran (average molecular weight 110,000), 1 mM EGTA and 4.5 mM MgCl₂. The plates were then kept at 4 °C, until more than 90% of the cells had become permeable to Trypan blue; this was routinely achieved after ~20 min for both myoblasts and myotubes; the kinetics were similar in the two sets of cells (data not shown). Permeabilization was monitored by studying plates set up in parallel. After permeabilization, the plates were transferred to a water bath at 25 °C, and the ADP-ribosyl transferase assay was started by the addition of 200 μl of a solution containing 200 mM HEPES pH 7.8, 750 mM KCl, 5 mM dithiothreitol, 4.5% (w/v) dextran, 1.35 M sucrose, 7.0 mM EGTA, 4.5 mM MgCl₂, 6.0 mM isonicotinic acid hydrazide, 18 mM NaF and 600 μM ³H-NAD (20 mCi mmol⁻¹). The enzyme incubation was continued for 10 min and then terminated by the addition of 2 ml of ice-cold 20% (w/v) trichloroacetic acid (TCA), containing 1% (w/v) nicotinamide and 2% (w/v) tetrasodium pyrophosphate. The acid-insoluble radioactivity was then estimated by scintillation counting. The DNA content of each sample was determined by the method of Kissane and Robins³³. The acid-insoluble radioactivity was demonstrated to be poly(ADP-ribose)³⁴. Incorporation was linear for at least 10 min at all stages of culture studied. The permeabilization procedure induced no detectable DNA strand breaks and at the end of the ADPRT assay the single-strand DNA molecular weight on alkaline sucrose gradients was the same as that in the untreated cells (results not shown). Total ADPRT activity was estimated by including a final concentration of 0.5% (v/v) Triton X-100 and 100 $\mu\text{g ml}^{-1}$ of DNase I in the assay mixture. ■, Physiological ADPRT activity; □, total potential ADPRT activity. The fraction of muscle cells fused (●) was determined as previously described²⁹. Each point in the figure is the mean of at least six measurements in three separate experiments; the bars represent the standard error of the mean. Percentage of fused myoblasts (●) and CPK activity (○) were measured as previously described³⁵. The rate of DNA synthesis (▲) was estimated by pulsing the cultures with 1 $\mu\text{Ci ml}^{-1}$ ³H-thymidine (1 Ci mmol⁻¹) for 1 h.

the onset of cell fusion, however, the physiological activity begins to rise, most of the increase taking place before 45 h. By 72 h, when ~70% of the cells have fused, the specific activity of ADPRT has increased threefold (Fig. 1). In contrast, the total potential ADPRT activity is higher than the physiological activity at all times and does not alter significantly (Fig. 1), implying that the increase in the physiological activity is due to enzyme activation, and not to an increase in the number of enzyme molecules. ADPRT activation precedes both cell fusion and the rise in CPK activity (Figs 1, 2b, d).

We have analysed the function of ADPRT by the use of enzyme inhibitors¹³. In the continuous presence of either 3-aminobenzamide or 3-methoxybenzamide²², myoblast fusion is inhibited in a concentration-dependent manner. 3-Aminobenzamide (8 mM) or 3-methoxybenzamide (5 mM) inhibit myoblast fusion essentially completely, myoblast proliferation is not inhibited (results not shown, but see Fig. 2). In contrast, the non-inhibitory analogues 3-aminobenzoate and 3-methoxybenzoate cause no significant inhibition of fusion at corresponding concentrations. Inhibitors of ADPRT block reversibly both myoblast fusion (Fig. 2a) and the usual increase in CPK activity (Fig. 2b). The replacement of 3-aminobenzamide-containing medium with medium from control cultures of the same age, at 44 or 54 h of culture, reversed the inhibition and cell fusion began 10 or 14 h later respectively (Fig. 2a). Removal of the inhibitor at 54 h of culture results in a rise in CPK activity 12 h later (Fig. 2b).

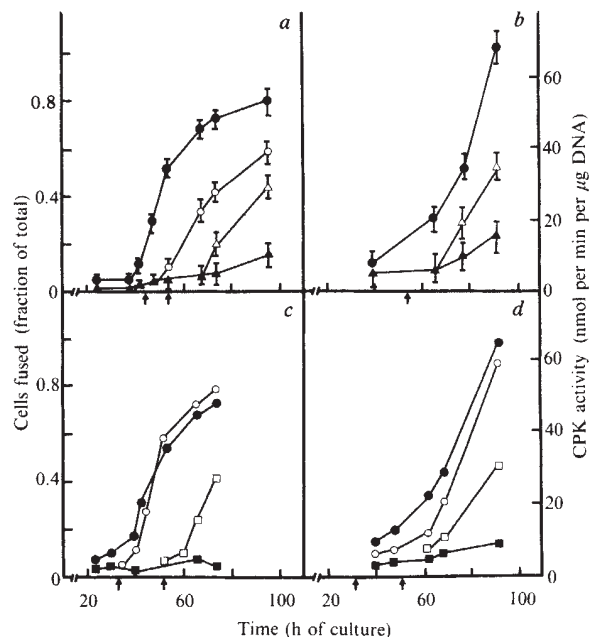


Fig. 2 Reversible inhibition of myoblast terminal differentiation. Reversible inhibition by 3-aminobenzamide (a, b) and by depletion of NAD levels (c, d). a and c show myoblast cell fusion; b and d show CPK activity. a, b, Muscle cells were prepared and cultured as described in Fig. 1 legend. ●, Control; ▲, continuous presence of 8 mM 3-aminobenzamide. The medium containing inhibitor was removed and replaced with medium from parallel control cultures of the same age at 44 h (○) or 54 h (△). Each point is the mean of six measurements in three separate experiments; the bars are the standard errors. CPK activity was estimated as previously described³⁵. c, d, Muscle cells were cultured in nicotinamide-free medium with dialysed serum and embryo extract. ■, No nicotinamide; 1 mM nicotinamide added at the time of plating (0 h ●), at 34(c) or 32(d) h of culture (○), or at 52 h of culture (□). Each point is the mean of at least two independent experiments. The arrows indicate the time at which the inhibitor was removed or nicotinamide returned in each experiment, as indicated above.

Because of a possible objection to the use of enzyme inhibitors, we also experimentally manipulated ADP-ribosylation by partially depleting the cells of their NAD content, by nicotinamide starvation¹³. After 44 h of culture in a nicotinamide-free medium the NAD level had fallen to $29 \pm 3\%$ (mean $\pm$ s.e.m., $n=6$ in two separate experiments). Nicotinamide (1 mM) can restore the control NAD level within 4.5 h. The NAD-depleted myoblasts do not fuse to form multinucleated fibres (Fig. 2c), nor do they show the usual rise in CPK activity (Fig. 2d). This block in cell differentiation is reversible; the re-addition of 1 mM nicotinamide to the depleted cultures at 32 or 52 h reverses the inhibition 6 or 8 h later respectively (Fig. 2c, d).

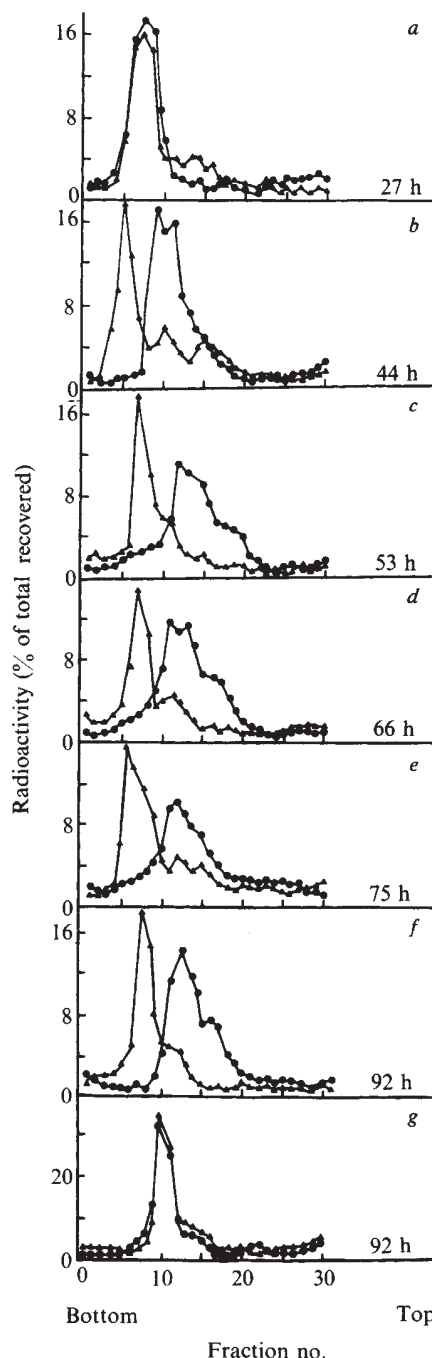
In several cases, in both prokaryotes and eukaryotes, alterations in gene activity involve changes in the DNA, including DNA modification, gene rearrangement and DNA transpositions^{23,24}. We might therefore expect DNA strand breaks to appear during certain types of change in gene expression and cytodifferentiation.

In alkaline sucrose gradients which estimate the size of single-stranded DNA, the DNA from proliferating cultures has a high molecular weight (Fig. 3a). At 44 h, when only 15% of the cells have fused (see Fig. 1), there is a significant reduction in the molecular weight of the cellular DNA (Fig. 3b). By 53 h the maximum number of single-strand breaks are present (Fig. 3c), although only 50–60% of the myoblasts have fused. The molecular weight then remains constant up to 92 h of culture (Fig. 3c–f). Only single-strand breaks appear in the DNA; there is no decrease in the molecular weight of the DNA in neutral sucrose gradients containing ionic detergents between 25 and 92 h of culture (Fig. 3g). We have been unable to establish what happens after 92 h of culture, so we do not yet know whether the DNA breaks persist or are subsequently rejoined. The experiments displayed in Fig. 3 are also 'mixing' experiments, and show that in our experimental conditions, mixing of early (without strand breaks) and late (with strand breaks) cells does not induce strand breaks in the DNA of early cells (see Fig. 3b–f).

Fig. 3 a–f, Molecular weight analysis of muscle cell DNA by alkaline sucrose gradients. Muscle cell cultures were radioactively labelled by the continuous presence of $1 \mu\text{Ci ml}^{-1}$ ^3H -thymidine, or of $0.2 \mu\text{Ci ml}^{-1}$ ^{14}C -thymidine. Before collecting the cells for the analysis of DNA molecular weight they were incubated in fresh medium for a further 3 h in the absence of radioactive nucleosides. The plates were washed twice in phosphate-buffered saline (PBS) containing 10 mM EDTA, and then cells were scraped off in the PBS-containing EDTA using a rubber policeman. 50 μl of ^3H -labelled and 50 μl of the ^{14}C -labelled cell suspensions, each containing the equivalent of $1-2 \times 10^4$ nuclei, were mixed and deposited in 400 μl of lysis buffer (100 mM NaOH, 100 mM NaCl, 10 mM EDTA, 0.2% (w/v) sarcosyl) over a 12-ml, 5–25% linear sucrose gradient containing 100 mM NaOH and 100 mM NaCl. The gradients were kept in the dark for 30 min at 20 °C, and then centrifuged for 2 h at 25,000 r.p.m. in a Beckman SW27 rotor. 400- μl fractions were collected on filter strips from the bottom of the gradients and the TCA-insoluble radioactivity estimated by scintillation counting. $\blacktriangle$, 27-h cultures labelled with ^{14}C -thymidine; $\bullet$, 44-, 53-, 66-, 75- and 92-h cultures labelled with ^3H -thymidine. g, Molecular weight analysis of muscle cell DNA by neutral sucrose gradients. Muscle cell cultures were radioactively labelled and collected as described above. The equivalent of $1-2 \times 10^4$ nuclei were deposited in 400 μl of lysis buffer (0.2% (w/v) sarcosyl, 0.08% (w/v) sodium deoxycholate, 1.5 M NaCl, 100 mM sodium citrate and 20 mM EDTA, pH 8.9) over a 12 ml 5–25% linear sucrose gradient containing 2.0 M NaCl, 10 mM sodium citrate and 1.0 mM EDTA, pH 8.9, over a cushion of 0.5 ml 60% sucrose. The gradients were kept in the dark for 1 h at 20 °C, and then centrifuged for 1 h at 20,000 r.p.m. in a Beckman SW27 rotor. 400- μl fractions were collected on filter strips from the bottom of the gradients and the TCA-insoluble radioactivity estimated by scintillation counting. $\blacktriangle$, 25-h culture labelled with ^{14}C -thymidine; $\bullet$, 92-h culture labelled with ^3H -thymidine.

Alkaline sucrose gradients detect single-strand breaks that are induced by the alkaline conditions of the gradients (for example, apurinic sites), as well as true single-strand breaks which had been generated enzymatically. We have distinguished between these two phenomena by examining the sedimentation rate of nucleoids²⁵. In neutral sucrose gradients containing 1.0 M NaCl, nucleoids from 24 and 38 h cultures sediment rapidly; in contrast, 6 h later, at 44 h, the nucleoids sediment more slowly, and by 68 h a considerable number of metabolically induced single-strand DNA breaks are evident (Fig. 4a). Increasing the salt concentration of the gradients to 1.95 M NaCl, and thus stripping the nuclei of yet more of their protein, does not alter the results significantly (Fig. 4a). Thus, the reduction in the sedimentation of the nucleoids is not due to changes in the protein composition of the chromatin.

This inference is also drawn from several experiments with ethidium bromide. In the presence of $5 \mu\text{g ml}^{-1}$ ethidium



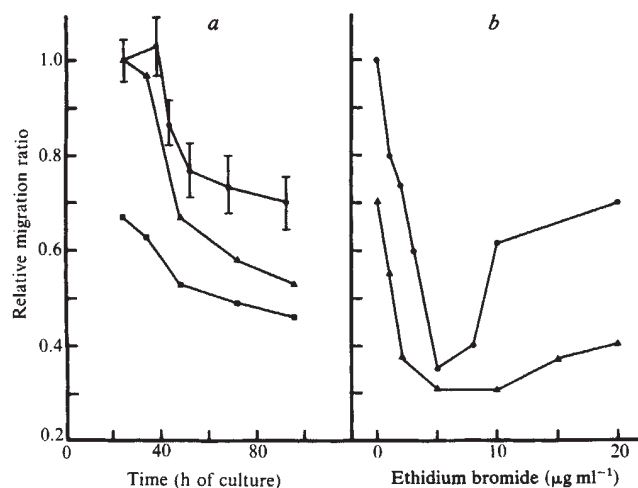


Fig. 4 *a*, The nucleoid sedimentation rate of muscle cells at different stages in culture. 50 μ l of muscle cells suspended in PBS (containing the equivalent of $1-5 \times 10^5$ nuclei) were deposited inside 150 μ l of lysis buffer (giving a final concentration of 2 mM EDTA, 0.5% (v/v) Triton X-100 and 100 mM Tris-(hydroxymethyl)-aminomethane pH 8.0) over a 4.6 ml 15–30% linear sucrose gradient containing 1 mM EDTA and 10 mM Tris-(hydroxymethyl)-aminomethane pH 8.0. The lysis mixture and the gradients also contained 1.0 M NaCl (●), 1.95 M NaCl (▲), or 1.95 M NaCl and 5 μ g ml $^{-1}$ ethidium bromide (■). The gradients were kept in the dark for 30 min at 20°C and then centrifuged at 10,000 r.p.m. in a Beckman SW50.1 rotor for 30 min. The gradients were collected from the bottom of the centrifuge tubes and pumped through a flow cell. The positions of the nucleoids were determined by measuring the absorbance at 254 nm, and are expressed as a fraction of the distance migrated by the nucleoids of 24-h cultures in the same conditions (●, ▲) or in 1.95 M NaCl (■). The error bars represent the standard error of the mean of at least three measurements. Nucleoids are residual nuclei stripped of most of their proteins by high-salt solutions and neutral detergents. Single-strand breaks in local domains of supercoiled DNA relieve the supercoiling, and lead to an expansion of the volume of the nucleoids and to a slower rate of sedimentation. *b*, The effect of ethidium bromide on the sedimentation rate of muscle cell nucleoids. Procedure as in *a* except that the lysis mixture and the gradients contained 1.95 M NaCl and the indicated concentration of ethidium bromide. Nucleoids from 24-h (●) or 72-h (▲) cultures were kept in the lysis mixture for only 15 min before being centrifuged at 10,000 r.p.m. for 45 min. The nucleoid sedimentation rate is expressed as a fraction of the distance migrated by nucleoids from 24-h cultures in the absence of ethidium bromide. The binding of intercalating agents, such as ethidium bromide, to DNA molecules under topological constraint produces a characteristic biphasic effect. With increasing concentrations of intercalating agents the number of superhelical turns are reduced and eventually a minimum value is reached. Increasing the concentration further introduces 'negative' turns in the DNA³⁶. The introduction of single-strand breaks in the local DNA domains eliminates the topological constraint and reduces the characteristic effect of the intercalating agent on the nucleoid as a whole. These changes in the extent of DNA supercoiling are reflected in the sedimentation rate of the nucleoids in neutral sucrose gradients³⁷.

bromide, the sedimentation of nucleoids from 24-h cultures is reduced from 1.0 to 0.67 (Fig. 4a). However, the same concentration of ethidium bromide has a much smaller effect on the sedimentation at 96 h, which is only reduced from 0.56 to 0.46. In nucleoids from 24-h cultures, increasing concentrations of ethidium bromide induce the biphasic effect characteristic of DNA molecules under topological constraints (Fig. 4b), whereas nucleoids from 72-h cultures are affected less (Fig. 4b), probably due to the presence of single-strand breaks. We have estimated the number of single-strand DNA breaks^{26–28} in differentiating muscle cell DNA, from 20 to 90 h (Fig. 5a–d); few single-strand breaks were detectable in the DNA at either

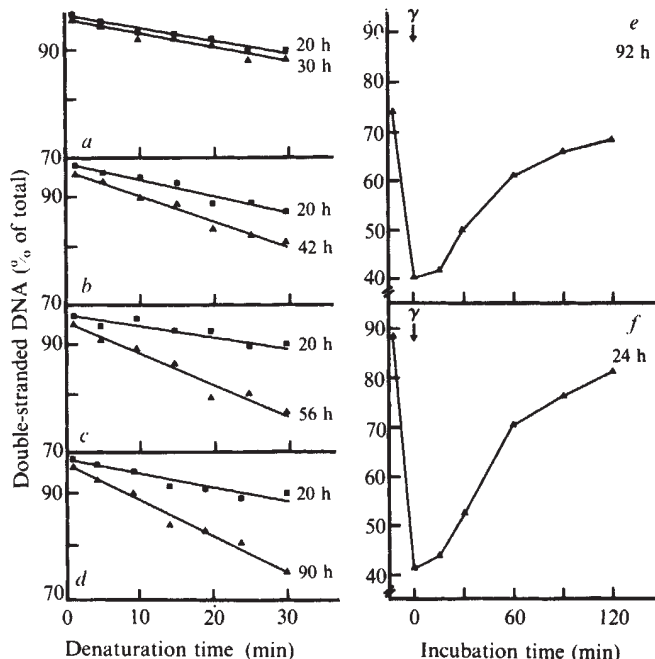


Fig. 5 *a–d*, Rate of DNA strand separation in alkali. The rate of DNA strand separation in alkali was measured with the Rydberg²⁷ modification of the Ahnström-Erixon alkaline denaturation and hydroxyapatite procedure^{26,28}. Briefly, DNA strand separation was initiated by the injection of 50 μ l of cell suspension containing the equivalent of $1-2 \times 10^4$ nuclei from a 20-h culture labelled with 14 C-thymidine (■) and $1-2 \times 10^4$ nuclei from 3 H-thymidine-labelled cultures at 30, 42, 56 or 90 h of culture (▲) into 1 ml of the alkaline solution containing 30 mM NaOH, 10 mM Na₂HPO₄ and 900 mM NaCl, pH 12.0. The injection was forceful to ensure adequate and immediate mixing of the cell suspension and the alkaline solution. This mixture was immediately placed in the dark and kept at 20°C for the required period. The DNA strand separation was terminated at intervals by the injection of 1 ml of ice-cold 34 mM HCl. Within 5 s of the neutralization, the solution was sonicated for 15 s. Before separating the single- and double-stranded DNA on hydroxyapatite columns, 80 μ l of 10% (w/v) SDS solution was added to each sample (~2.0 ml) to obtain a final SDS concentration of 0.4%. Samples were then loaded on 5 $\times$ 30 mm hydroxyapatite columns equilibrated with 10 mM potassium phosphate buffer, pH 6.9. The unbound nucleotides were eluted with 2 ml of 10 mM potassium phosphate buffer and the single- and double-stranded DNA was eluted with 3 $\times$ 0.5 ml of 125 mM and 400 mM phosphate buffer respectively. The total radioactivity loaded, and the single- and double-stranded DNA recovered, were measured by scintillation counting. A standard curve was established by exposing 24-h myoblast cultures to 50–300 rad of γ -radiation (results not shown). These cultures have accumulated very few endogenous single-strand breaks at this time. The logarithm of the percentage of double-stranded DNA remaining after 30 min in alkali declines linearly with the applied dose of radiation. Chick muscle cell DNA as a molecular weight of $\sim 10^{12}$, calculated from a diploid DNA content of 2.5 pg per nucleus. We estimate that approximately two single-strand breaks are formed per 10^{12} daltons per rad³⁸. We can now convert the percentage of double-stranded DNA remaining to the number of single-strand breaks per 10^{12} daltons. *e, f*, Rate of DNA repair following γ -radiation. 24- and 92-h muscle cell cultures were irradiated with 300 rad of γ -radiation. The rate of strand-rejoining was estimated by measuring the rate of DNA strand separation in alkali following the re-incubation of the irradiated cells in fresh medium for the indicated times.

20 or 30 h, but by 42 h approximately 50–100 single-strand breaks per 10^{12} daltons (per diploid genome) had appeared. By 56 h they had increased to 100–300 per genome (Fig. 5c), and did not increase thereafter (Fig. 5d).

The presence of DNA strand breaks in differentiating cells may be attributed to a general deficiency of DNA repair.

However, these differentiating muscle cells are proficient in repair and can readily rejoin single-strand breaks. There is no detectable decline in the ability to rejoin DNA strand breaks caused by γ -irradiation (Fig. 5e, f). In the 92-h cultures (Fig. 5e), a significant number of DNA strand breaks exist before irradiation; exposure to 300 rads of γ -radiation would be expected to introduce a further 600 breaks, about twice as many as were metabolically engendered. The rate at which these breaks were rejoined was the same as that in 24-h myoblasts—but rejoining stopped when the number of breaks had dropped to the endogenous level. This implies that these endogenous breaks are retained in the face of a proficient DNA strand-rejoining capacity, perhaps because they are of a special character or occur at specific nucleotide sequences; alternatively, there may be a shift in the steady-state level of breaks.

Our observations have been confirmed and extended in subsequent studies showing that ADPRT activity is required for the differentiation of the protozoan *Trypanosoma cruzi* (G. T. Williams, personal communication), the stimulation of resting circulating human lymphocytes²⁹ and the cytodifferentiation of hepatocytes³⁰. The involvement of DNA strand breaks in some categories of cellular differentiation may be the molecular basis for the requirement of ADPRT in cytodifferentiation.

Thus we propose that a reduction in the single-strand molecular weight of the DNA and the consequent obligatory increase in the intrinsic, physiological ADPRT activity are integral and early events in the cellular differentiation of primary chick myoblast cultures. The single-strand breaks may signify some underlying mobility of the genome. The results reported here are consistent with the notion that nuclear ADPRT participates commonly in situations where nicking and rejoining of DNA occur.

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1. Sugimura, T. *Prog. Nucleic Acid Res. molec. Biol.* **13**, 127–151 (1973).
2. Hilz, H. & Stone, P. R. *Rev. Physiol. Biochem. Pharmac.* **76**, 1–59 (1976).
3. Hayaishi, O. & Ueda, K. A. *Rev. Biochem.* **46**, 95–116 (1977).
4. Purnell, M. R., Stone, P. R. & Whish, W. J. D. *Biochem. Soc. Trans.* **8**, 215–227 (1980).
5. Smulson, M. E. & Sugimura, T. (eds) *Novel ADP-Ribosylations of Regulatory Enzymes and Proteins* (Elsevier, Amsterdam, 1980).
6. Tsopanakis, C., Leeson, E., Tsopanakis, A. & Shall, S. *Eur. J. Biochem.* **90**, 337–345 (1978).
7. Janakidevi, K. & Koh, C. *Biochemistry* **13**, 1327–1330 (1974).
8. Miller, E. G. *Biochim. biophys. Acta* **395**, 191–200 (1975).
9. Halldorsson, H., Gray, D. A. & Shall, S. *FEBS Lett.* **85**, 349–352 (1978).
10. Berger, N. A., Weber, G. & Kaichi, A. S. *Biochim. biophys. Acta* **519**, 89–104 (1978).
11. Benjamin, R. G. & Gill, D. M. *J. biol. Chem.* **255**, 10493–10508 (1980).
12. Durkacz, B. W., Nduka, N., Omidiji, O., Shall, S. & Zia'ee, A. A. in *Novel ADP-Ribosylations of Regulatory Enzymes and Proteins* (eds Smulson, M. E. & Sugimura, T.) 207–216 (Elsevier, Amsterdam, 1980).
13. Durkacz, B. W., Omidiji, O., Gray, D. A. & Shall, S. *Nature* **283**, 593–596 (1980).
14. Creissen, D. & Shall, S. *Nature* **296**, 271–272 (1982).
15. Caplan, A. I. & Rosenberg, M. J. *Proc. natn. Acad. Sci. U.S.A.* **72**, 1852–1857 (1975).
16. Claycomb, W. C. *Biochem. J.* **154**, 387–393 (1976).
17. Rastl, E. & Swetly, P. J. *Biol. Chem.* **253**, 4333–4340 (1978).
18. Farzaneh, F. & Pearson, C. K. *Dev. Biol.* **72**, 254–265 (1979).
19. Jackowski, G. & Kun, E. *J. biol. Chem.* **256**, 3667–3670 (1981).
20. Bredehorst, R., Wilckens, K., Adamietz, P., Steinhagen-Thiessen, E. & Hilz, H. *Eur. J. Biochem.* **120**, 267–274 (1981).
21. Farzaneh, F., Shall, S. & Zalin, R. in *Novel ADP-Ribosylations of Regulatory Enzymes and Proteins* (eds Smulson, M. E. & Sugimura, T.) 217–225 (Elsevier, Amsterdam, 1980).
22. Purnell, M. R. & Whish, W. J. D. *Biochem. J.* **185**, 775–777 (1980).
23. Nevers, P. & Saedler, H. *Nature* **268**, 109–115 (1977).
24. Robertson, M. *Nature* **297**, 184–186 (1982).
25. Cook, P. E., Brazell, I. A. & Jost, E. *J. Cell Sci.* **22**, 303–324 (1976).
26. Ahnström, G. & Erikson, K. in *DNA Repair: A Laboratory Manual of Research Procedures* (eds Friedberg, E. C. & Hanawalt, P. C.) 403–418 (Dekker, New York, 1981).
27. Rydberg, B. *Radiat. Res.* **61**, 274–287 (1975).
28. Ahnström, G. & Erikson, K. *Int. J. Radiat. Biol.* **23**, 285–289 (1973).
29. Johnston, A. P. & Williams, G. T. *Nature* **300**, 368–370 (1982).
30. Althaus, F. R. *et al. Nature* **300**, 366–368 (1982).
31. Nguyen Thi Man, Morris, G. E. & Cole, R. J. *Dev. Biol.* **47**, 81–96 (1975).
32. Reinhard, P., Burkhalter, M. & Gautschi, J. R. *Biochim. biophys. Acta* **474**, 500–511 (1977).
33. Kissane, J. M. & Robins, E. *J. biol. Chem.* **233**, 184–188 (1958).
34. Farzaneh, F. & Pearson, C. K. *Biochem. biophys. Res. Commun.* **84**, 537–543 (1978).
35. Zalin, R. *J. Dev. Biol.* **53**, 1–9 (1976).
36. Bauer, W. & Vinograd, J. *J. molec. Biol.* **33**, 141–171 (1968).
37. Cook, P. R. & Brazell, I. A. *J. Cell. Sci.* **22**, 287–302 (1976).
38. Ormerod, M. G. & Stevens, U. *Biochim. biophys. Acta* **232**, 72–82 (1971).

Effects of altered [ADP-ribose]_n metabolism on expression of fetal functions by adult hepatocytes

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The nuclear enzyme ADP-ribosyltransferase (ADPRT) catalyses the postsynthetic modification of chromatin proteins¹. The significance of [ADP-ribose]_n-modified chromatin proteins for the regulation of chromatin function is not understood¹. Recently, [ADP-ribose]_n biosynthesis has been demonstrated to participate in the repair of some types of DNA damage^{2–4}. Here we demonstrate that another function of [ADP-ribose]_n biosynthesis is related to the regulation of the expression of two fetal functions of cultured hepatocytes—the K-type (III) isozyme of pyruvate kinase, and γ -glutamyltranspeptidase, which is also a marker of early neoplastic transformation of liver cells⁵.

An involvement of ADP-ribosylation in cellular differentiation has been inferred from the observation of a temporal relationship between alterations in ADPRT activity and engagement of cells in terminal differentiation^{6–11}. However, [ADP-ribose]_n might primarily regulate the proliferative activity^{12,13} of cells undergoing terminal differentiation. It has therefore not been possible to ascribe definitively the expression of individual cellular functions during differentiation to changes in ADP-ribosylation activity¹. The recent introduction of 3-aminobenzamide, the most specific inhibitor of ADPRT known, has made it possible to determine directly the potential involvement of this enzyme in a particular biological process^{2,3}. We have used this inhibitor to determine the role of ADPRT in the expression of fetal functions by adult rat hepatocytes in primary monolayer culture.

We found that in rat hepatocytes cultured on collagen gel-nylon meshes¹⁴, ADPRT activity sharply increased to a maximum after 3 days in culture and then decreased to the low levels seen in freshly isolated hepatocytes (Fig. 1a). Coincident with the decrease in ADPRT activity, the activities of γ -glutamyltranspeptidase and the fetal (K-type) isozyme of pyruvate kinase sharply increased (Fig. 1b). These two enzyme activities are normally high in fetal hepatocytes *in vivo* but disappear shortly after birth^{15,16}. Previous studies in cultured hepatocytes have clearly shown that the expression of fetal pyruvate kinase isozyme activity is the result of induction of the isozyme protein¹⁶. We observed that induction of the fetal pyruvate kinase isozyme was totally inhibited in hepatocyte cultures treated with 3-aminobenzamide (10 mM) or nicotinamide (25 mM), two inhibitors of ADPRT (Fig. 2); this indicates that induction of the isozyme and alterations in ADP-ribosylation activity (Fig. 1) are causally related. The specificity of action³ of the ADPRT inhibitors was indicated by the inability of the non-inhibitory analogue², 3-aminobenzoic acid (1–10 mM), to prevent the expression of the fetal pyruvate kinase isozyme (Fig. 2a). In addition, the dose-response curves and the apparent K_i s for the actions of 3-aminobenzamide and nicotinamide on ADPRT and pyruvate kinase in intact hepatocytes were very similar⁴. When hepatocytes were maintained for 2–7 days in medium containing 10 mM aminobenzamide or 25 mM nicotinamide, a 70–95% inhibition of ADPRT activity was observed. This inhibition was reversible,

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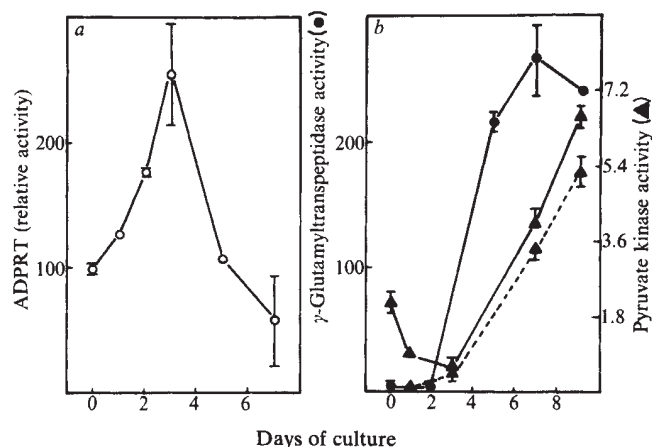


Fig. 1 Alterations in the activities of ADPRT, γ -glutamyltranspeptidase and pyruvate kinase of adult rat hepatocytes cultured on collagen gel-nylon meshes. *a*, Hepatocytes were isolated from male Holtzman rats (200–250 g, fed *ad libitum*) and cultured on collagen gel-nylon meshes as described previously¹⁴. At the times indicated, hepatocytes were recovered from the substratum by brief collagenase treatment¹⁴ and ADPRT activity measured as described elsewhere⁴ using ^3H -NAD as substrate in cells made permeable with digitonin²³. All activities are expressed relative to the activity of freshly isolated hepatocytes. Separate experiments indicated that the ADPRT activities measured as ^3H -NAD incorporation into hepatocytes following gentle permeabilization with digitonin²³ reflect the physiological enzyme activity of intact cells. Thus, a much higher proportion of $[2,8\text{-}^3\text{H}]$ adenosine radioactivity added to the medium of intact hepatocytes was found to be covalently linked to chromatin proteins after a labelling period (3-h pulse) that coincided with high ADPRT activity (day 3 of culture) (Fig. 1) compared to an identical labelling period immediately after plating of cells. This incorporation of $[2,3\text{-}^3\text{H}]$ adenosine radioactivity was inhibited in hepatocytes cultured in the presence of 3-aminobenzamide (10 mM) or nicotinamide (25 mM). *b*, Activities of γ -glutamyltranspeptidase (●—●) and pyruvate kinase (▲—▲) were determined as described^{14,16}. ▲—▲, Fetal (K-type) isozyme activity of pyruvate kinase. γ -Glutamyltranspeptidase activity is expressed as nmol *p*-nitroaniline formed per μg DNA per h¹⁴ and pyruvate kinase activity reflects the change in absorbance at 340 nm per μg DNA per h¹⁶. Bars indicate mean $\pm$ s.d. of 3–5 measurements in separate cell batches; symbols without bars represent the averaged values from two determinations in two separate cell batches.

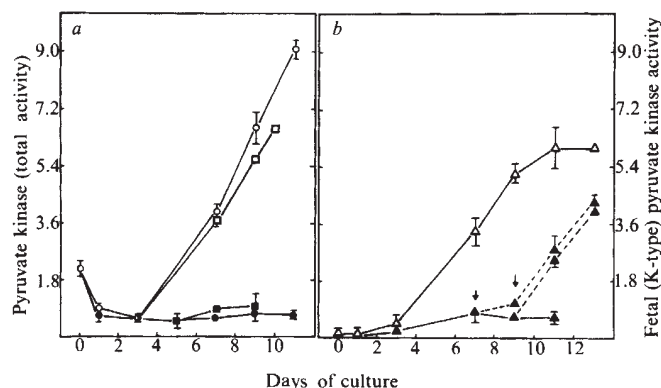


Fig. 2 Effects of the ADPRT inhibitors, 3-aminobenzamide (■, 10 mM), nicotinamide (●, 25 mM), and the non-inhibitory analogue 3-aminobenzoic acid (□, 10 mM), on the expression of pyruvate kinase activity by hepatocytes cultured on collagen gel-nylon meshes. *a*, Total pyruvate kinase activity of hepatocytes maintained in the presence of the above and in control cultures (○). *b*, Activity of the fetal (K-type) isozyme of pyruvate kinase in untreated control (△—△) and nicotinamide-treated (▲—▲) hepatocyte cultures. ▲—▲, Fetal isozyme activity after withdrawal (arrows) of nicotinamide from the culture medium. Bars indicate mean $\pm$ s.d. of 3–5 separate cell preparations; symbols without bars represent the averaged values from two determinations in two separate cell batches. None of the ADPRT inhibitors had any direct effect on pyruvate kinase activity *in vitro*.

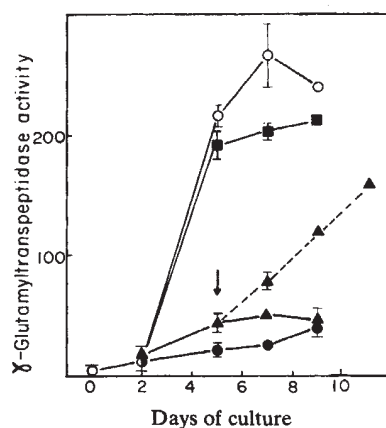


Fig. 3 Effect of nicotinamide (▲—▲, 25 mM), 3-aminobenzamide (●—●, 10 mM) and 3-aminobenzoic acid (■—■, 10 mM) on the expression of γ -glutamyltranspeptidase activity by hepatocytes cultured on collagen gel-nylon meshes. ○—○, Enzyme activities of untreated control cultures. ▲—▲, Enzyme activity after withdrawal (arrow) of nicotinamide from the culture medium. Inhibition of γ -glutamyltranspeptidase expression was also observed with other ADP-ribosylation inhibitors such as isonicotinamide, benzamide and theophylline⁴. Results expressed as mean $\pm$ s.d. of 3–5 separate measurements; symbols without bars represent the averaged values from two determinations in two separate cell batches. None of the ADPRT inhibitors had any direct effect on γ -glutamyltranspeptidase activity *in vitro*.

as was the repression of the fetal pyruvate kinase isozyme (Fig. 2b).

We then investigated the possibility that ADP-ribosylation might have a coordinative function in the expression of hepatocellular functions *in vitro*. We studied the effect of 3-aminobenzamide and nicotinamide on the expression in cultured hepatocytes of albumin, α -fetoprotein and γ -glutamyltranspeptidase¹⁴. The expression of γ -glutamyltranspeptidase activity was suppressed by this treatment (Fig. 3), whereas the expression of α -fetoprotein and albumin was unaffected (data not shown) as judged by immunological quantification of these proteins using monospecific antibodies¹⁴. The inhibition of γ -glutamyltranspeptidase expression required the presence of ADP-ribosylation inhibitors during the first 5 days of culture. Treatment of hepatocytes after day 5 of culture, that is, when ADPRT activity had returned to low levels (Fig. 1), caused only marginal inhibition (~10%) of this expression. The specificity of action for ADPRT inhibitors was indicated by the inability of the non-inhibitory analogue², 3-aminobenzoic acid (1–10 mM), to inhibit the expression of γ -glutamyltranspeptidase activity (Fig. 3). A histochemical study¹⁷ revealed that γ -glutamyltranspeptidase was uniformly suppressed in all hepatocytes of a culture dish, indicating that the actions of these ADPRT inhibitors were not selective for a small cell population within the liver (data not shown). In addition, the effect of these inhibitors on γ -glutamyltranspeptidase expression was reversible (Fig. 3).

Nicotinamide has been reported to inhibit general protein synthesis during the first 24 h of hepatocyte culture¹⁸. Although we could confirm this observation, we found that both nicotinamide (25 mM) and 3-aminobenzamide (10 mM), stimulated ^3H -leucine incorporation into total hepatocellular proteins after this initial culture period. Therefore, we conclude that the effects of these two ADPRT inhibitors on the expression of pyruvate kinase and γ -glutamyltranspeptidase are not due to a general inhibition of protein synthesis. Similarly, previous studies from our laboratory demonstrated the independence of the expression of fetal functions by cultured hepatocytes from DNA synthesis activity¹⁴, which increases after 2–3 days in culture in the absence of measurable cell replication. Although this increase in DNA synthesis was slightly inhibited by

nicotinamide (25 mM), continuous treatment of hepatocyte cultures for up to 9 days with 3-aminobenzamide did not affect this activity (data not shown). Thus, the action of ADPRT inhibitors on pyruvate kinase expression by the hepatocytes was not related to effects on DNA synthesis. These data also suggest that the presumed specificity of some ADPRT inhibitors might not be absolute. Similarly, effects on extranuclear ADP-ribosylation reactions cannot be totally excluded.

There is increasing evidence that ADPRT activity is largely dormant in the cell nucleus unless factors inducing DNA fragmentation are manifest^{1,19-21}. In general, DNA fragmentation presumably then initiates the activation of ADPRT. To test this, we examined hepatocellular DNA for the presence of DNA strand breaks in relation to changes in ADP-ribosylation. Using a slight modification⁴ of the sensitive technique of Birnboim and Jevcak²², we were unable to find an increase in the number of DNA strand breaks either before or concomitant with changes in ADPRT activity (Fig. 1) although we observed a gradual but slow increase in the number of DNA strand

breaks with increasing time in culture. This increase was unaffected by 3-aminobenzamide or nicotinamide (data not shown). These results suggest that the transient and spontaneous alterations in ADPRT activity seen in primary cultures of hepatocytes (Fig. 1) are not causally related to DNA fragmentation, which agrees with the observation of Jackowski and Kun⁶ who found age-dependent variations in the ADPRT activity of cardiocyte nuclei in the absence of measurable DNA fragmentation.

Our results show that [ADP-ribose]_n metabolism is involved in the regulation of the expression of two fetal functions by adult hepatocytes in primary culture. It will be interesting to determine whether carcinogen-induced alterations in cellular [ADP-ribose]_n metabolism⁷ are related to some of the well known functional phenotypic changes associated with neoplastic transformation^{5,15}.

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1. Purnell, M. R., Stone, P. R. & Whish, W. J. D. *Biochem. Soc. Trans.* **8**, 215-227 (1980).
2. Durkacz, B. W., Omidiji, O., Gray, D. A. & Shall, S. *Nature* **283**, 593-596 (1980).
3. Creissen, D. & Shall, S. *Nature* **296**, 271-272 (1982).
4. Althaus, F. R., Lawrence, S. D., Sattler, G. L. & Pitot, H. C. *J. biol. Chem.* **257**, 5528-5535 (1982).
5. Pitot, H. C. & Sirica, A. E. *Biochim. biophys. Acta* **605**, 191-215 (1980).
6. Jackowski, G. & Kun, E. *J. biol. Chem.* **256**, 3667-3670 (1981).
7. Porteous, J. W., Furneaux, H. M., Pearson, C. K., Lake, C. M. & Morrison, A. *Biochem. J.* **180**, 455-463 (1979).
8. Savard, P., Aubin, R., Whish, W. J. D., Lord, A. & Poirier, G. *Cancer Res.* **41**, 1417-1421 (1981).
9. Pekala, P. H., Lane, M. D., Watkins, P. A. & Moss, J. *J. biol. Chem.* **256**, 4871-4876 (1981).
10. Bredehorst, R., Wilckens, K., Adamietz, P., Steinhagen-Thiessen, E. & Hilz, H. *Eur. J. Biochem.* **120**, 267-274 (1981).
11. Gartemann, A., Bredehorst, R., Wielckens, K., Stratling, W. H. H. & Hilz, H. *Biochem. J.* **198**, 37-44 (1981).

12. Berger, N. A., Weber, G. & Kaicchi, A. S. *Biochim. biophys. Acta* **519**, 87-104 (1978).
13. Berger, N. A., Adams, J. W., Sikorski, G. W., Petzold, S. J. & Shearer, W. T. *J. clin. Invest.* **62**, 111-118 (1978).
14. Sirica, A. E., Richards, W., Tsukada, Y., Sattler, C. A. & Pitot, H. C. *Proc. natn. Acad. Sci. U.S.A.* **76**, 283-287 (1979).
15. Pitot, H. C. & Sirica, A. E. in *Differentiation and Neoplasia* (eds McKinnell, R. G., DiBerardino, M. A., Blumenfeld, M. & Bergad, R. D.) 241-250 (Springer, Berlin, 1980).
16. Leffert, H. et al. *Proc. natn. Acad. Sci. U.S.A.* **75**, 1834-1838 (1978).
17. Rutenberg, A. M. et al. *J. Histochem. Cytochem.* **17**, 517-526 (1969).
18. Villa, P., Hockin, L. J. & Paine, A. J. *Biochem. Pharmacol.* **29**, 1773-1777 (1980).
19. Tanaka, Y., Hashida, T., Yoshihara, H. & Yoshihara, K. *J. biol. Chem.* **254**, 12433-12438 (1979).
20. Benjamin, R. C. & Gill, D. M. *J. biol. Chem.* **255**, 10493-10501, 10502-10508 (1980).
21. Ogata, N., Uedo, K., Kawauchi, M. & Hayaishi, O. *J. biol. Chem.* **256**, 4135-4137 (1981).
22. Birnboim, H. C. & Jevcak, J. J. *Cancer Res.* **41**, 1889-1892 (1981).
23. Fiskum, G., Craig, S. W., Decker, G. L. & Lehninger, A. L. *Proc. natn. Acad. Sci. U.S.A.* **77**, 3430-3434 (1980).

Role of DNA breaks and ADP-ribosyl transferase activity in eukaryotic differentiation demonstrated in human lymphocytes

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ADP-ribosyl transferase (ADPRT) is a DNA-dependent nuclear enzyme which covalently attaches ADP-ribose moieties derived from NAD to proteins to form mono- or poly-ADP-ribosyl derivatives^{1,2}. ADPRT activity is strongly stimulated by breaks in DNA^{3,4} and the enzyme is required for efficient DNA repair⁵⁻⁷. Several reports have suggested that the metabolism of poly- or mono-(ADP-ribose) might also be involved in differentiation in various eukaryotes^{1,2,8-12}. In the most convincing of these, inhibitors of ADPRT were shown to block the differentiation of chick myoblasts *in vitro*^{11,12}, and differentiation was accompanied by the appearance of single-strand breaks in DNA. We present here evidence that ADPRT activity is obligatory early in the mitogen-induced activation of human peripheral blood lymphocytes. The requirement for this enzyme coincides with a rapid (1-8 h) rejoining of single-strand breaks present in the DNA of quiescent lymphocytes. Hence, DNA breaking and rejoining, regulated by ADPRT, may be involved in a general mechanism of differentiation.

Quiescent lymphocytes can be stimulated to differentiate by various polyclonal activators, or 'mitogens', which mimic the effect of specific antigens on individual cells¹³⁻¹⁵. This differentiation involves a change in morphology, initiation of proliferation and acquisition of various effector functions. In the present study human peripheral blood lymphocytes, comprising

~70% T cells, were activated by three mitogens: pokeweed mitogen (PWM), concanavalin A (Con A) and *Phaseolus vulgaris* phytohaemagglutinin (PHA)¹³.

The effect of several inhibitors of ADPRT, that is, benzamide¹⁶, 3-aminobenzamide¹⁷, 3-methoxybenzamide¹⁷ and 5-methylnicotinamide¹⁸, on the mitogen-stimulated incorporation of ³H-thymidine was studied. If they were added slightly before or at the same time as the mitogen, all of the inhibitors significantly decreased the response to all three mitogens; 3-methoxybenzamide and 5-methylnicotinamide were the most effective (Fig. 1). Conversely, if the inhibitors were added to the cells 24 h after PHA or Con A, before any DNA synthesis had occurred, a substantial response was observed (Fig. 1b, c). The incorporation of ³H-thymidine at day 2 was near to normal and only the cells responding more slowly and synthesizing DNA at days 3 and 4 were substantially affected by this later addition of inhibitors.

At the concentrations used, the inhibitors did not affect lymphocyte viability, assessed by exclusion of eosin, over a period of 7 days in culture. Similarly, they did not inhibit the lymphoid cell cycle in the absence of differentiation as evidenced by their lack of significant effect on the proliferation in culture of previously stimulated lymphoblasts (Fig. 1b, c), human T lymphoblastoid cell lines Molt 4 and CEM (unpublished observations) and murine leukaemic cells L1210¹⁹.

To define more precisely the time during their response at which the cells are susceptible to inhibition, lymphocytes were stimulated with PHA or Con A, and 3-methoxybenzamide or 5-methylnicotinamide was added to the cultures at various times. Figure 2 shows the response observed at day 2. Within 1 h of PHA stimulation, a significant proportion (~20%) of the cells synthesizing DNA at day 2 had escaped from the inhibitable stage and by 12-16 h a plateau was reached when 60-70% of cells had passed through the phase at which they were susceptible to inhibition. The time of addition of inhibitors which corresponded to a response at day 2 of 50% of normal was 15.1 ± 2.3 h for Con A and 8.0 ± 4.2 h for PHA. This is

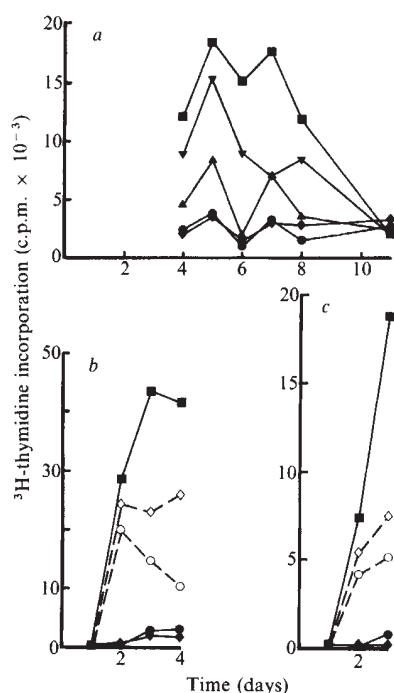


Fig. 1 Effect of inhibitors of ADPRT on lymphocyte transformation. Mononuclear leukocytes, comprising 95% lymphocytes, were isolated from human peripheral blood²⁸ and incubated at 10^6 cells ml^{-1} at 37°C in an atmosphere of 5% CO_2 in RPMI 1640 medium containing 25% human AB serum and 1/250 dilution of PWM (Gibco) (a), 5% fetal calf serum and $2.5 \mu\text{g ml}^{-1}$ PHA leucoagglutinin (Pharmacia) (b), or 5% fetal calf serum and $1 \mu\text{g ml}^{-1}$ Con A (Sigma) (c). The cells were incubated with medium containing no inhibitor (—■—), 2.5 mM benzamide (—▲—), 5 mM 3-aminobenzamide (—△—), 2.5 mM 3-methoxybenzamide (—●—) or 5 mM 5-methylnicotinamide (—○—) from 30 min before the addition of mitogen. Alternatively, freshly dissolved 2.5 mM 3-methoxybenzamide (—○—) or 5 mM 5-methylnicotinamide (—○—) was added to the cultures 24 h after addition of mitogen. [$6\text{-}^3\text{H}$]thymidine (5 Ci mmol^{-1} ; Amersham) was added to a final concentration of $2 \mu\text{Ci ml}^{-1}$ for the final 6 h before the cells were collected onto Whatman GF/C paper and washed with water using a cell harvester at the times indicated after mitogen stimulation. Each point represents the mean c.p.m. incorporated into DNA of duplicate 200- μl cultures. Control cultures without mitogen incorporated 300–1,000 c.p.m. The time course of the PWM response is invariably slower than that of the other two mitogens.

consistent with the slower response to Con A compared with PHA in our system (Fig. 1).

ADPRT is involved in increasing DNA ligase II activity⁷ and so the depression of the lymphocyte response by inhibitors of ADPRT suggests that ligation of DNA might be an obligatory early event during lymphocyte activation. To test for this, the migration of lymphocyte 'nucleoids' on sucrose density gradients was observed at various times after mitogen stimulation (Fig. 3). Nucleoids are prepared from eukaryotic cells by lysis in a non-ionic detergent in the presence of high salt and EDTA^{19,20}. They have lost most of the nuclear protein and consist of DNA in a supercoiled conformation. Any breaks in DNA decrease the constraints on the supercoiled structure, allowing it to expand and causing the nucleoid to migrate more slowly in the density gradient. Determination of the nucleoid sedimentation rate is therefore a very sensitive method for detecting single-strand breaks^{21,22}.

Nucleoids from PHA-stimulated lymphocytes sedimented faster through the density gradients than those derived from quiescent lymphocytes. This difference could be detected as early as 1 h after stimulation and was maximal after about 8 h (Fig. 3). The change in sedimentation rate was much slower if 3-methoxybenzamide or 5-methylnicotinamide was included in

the culture medium (Fig. 3, compare e with f in expt 1; compare c with h, and e with i in expt 2). The increased rate of sedimentation of nucleoids from activated lymphocytes demonstrated that single-strand breaks present in the DNA of resting lymphocytes are rapidly rejoined after PHA stimulation. This early ligation of DNA breaks may be directly involved in inducing the expression of new genes responsible for the programmed activation of lymphocytes. Other workers have reported that lymphocyte nucleoids sediment much more slowly than those from most other cells^{19,20,22}, but the existence of DNA breaks in quiescent lymphocytes was unexpected and intriguing.

There is a close temporal correlation during lymphocyte activation between DNA strand rejoining and susceptibility to blocking of the response by ADPRT inhibitors (compare Figs 2 and 3). This observation, together with the inhibition of strand rejoining by inhibitors of ADPRT (Fig. 3) and the established role of ADPRT in DNA repair^{5-7,21}, suggests that this enzyme controls the rejoining of DNA strand breaks early in differentiation.

Recently, ADPRT activity has been demonstrated in membranes and implicated, via adenylate cyclase, in the hormonal regulation of certain tissues²³. It is conceivable that the chemicals we have used produce their effects by inhibiting the membrane enzyme rather than the nuclear enzyme. However, as mitogen stimulation of lymphocytes, unlike the effect of some polypeptide hormones, cannot be attributed simply to adenylate cyclase activity¹³, we consider this possibility unlikely.

ADPRT activity and the formation of single-strand breaks in DNA have previously been correlated with differentiation in chick embryo myoblasts^{11,12}. In addition, the morphological differentiation of the protozoan parasite, *Trypanosoma cruzi*, can be blocked by inhibitors of ADPRT (manuscript in preparation). Most importantly, the enzyme activity does not seem to be involved in cell cycle processes because its inhibition does not affect proliferation in the absence of differentiation in any of the three systems. This illustrates the operational specificity of the inhibitors. The concordant results obtained in these diverse systems support the hypothesis that ADPRT and transient DNA breaks are widely involved in the differentiation of eukaryotic cells.

It is possible that these events are involved in a specific rearrangement of genetic material as a ubiquitous mechanism for coherently altering gene expression during differentiation. Indeed, recent data suggest that there are some differences in

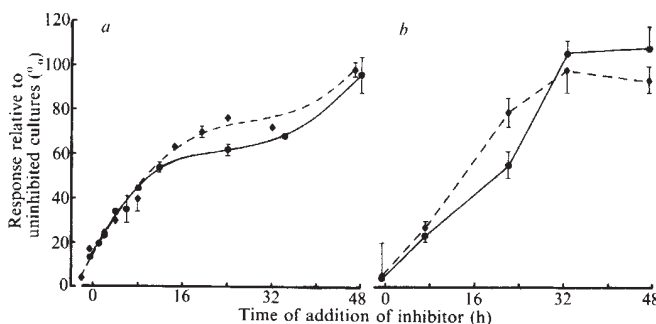


Fig. 2 Kinetics of susceptibility to inhibition during lymphocyte activation. In four separate experiments, lymphocytes were isolated from four individuals and cultured with PHA (a) or Con A (b) at time zero as described for Fig. 1. 2.5 mM 3-methoxybenzamide (—■—) or 5 mM 5-methylnicotinamide (—●—) was added to the cultures at the times indicated before or after addition of mitogen. The cultures were pulsed with ^3H -thymidine at 48 h and collected at 52 h as described for Fig. 1. The results are expressed as a percentage of the response (c.p.m. incorporated into DNA) in control cultures to which medium was added at the same time as inhibitor solution was added to the test cultures; 100% response represents 25,000–35,000 c.p.m. for PHA and 5,000–10,000 c.p.m. for Con A. Each point represents the mean $\pm$ s.e.m. for triplicate 200- μl cultures.

the arrangement of genetic material between different tissues of an individual person²⁴. The controlled appearance of temporary breaks in the DNA backbone could also be required to relax the tightly packed chromatin structure in particular areas, thus allowing the transcription of new genes²⁵⁻²⁷.

In addition to the exciting theoretical concepts outlined above, our present report suggests that chemicals which inhibit ADPRT activity may have several practical clinical uses. For example, if mitogen-induced activation of lymphocytes *in vitro* is indeed a valid model for antigenic stimulation of the immune system *in vivo*, the inhibitors should specifically block new immune responses while allowing established ones to continue. This would be very useful for many applications requiring immunomodulation, in particular during tissue transplantation.

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1. Hayaishi, O. & Ueda, K. A. *Rev. Biochem.* **46**, 95-116 (1977).
2. Purnell, M. R., Stone, P. R. & Whish, W. J. D. *Biochem. Soc. Trans.* **8**, 215-227 (1980).
3. Halldorsson, H., Gray, D. A. & Shall, S. *FEBS Lett.* **85**, 349-352 (1978).
4. Benjamin, R. & Gill, M. J. *biol. Chem.* **255**, 10493-10508 (1980).
5. Durkacz, B. W., Omidiji, O., Gray, D. A. & Shall, S. *Nature* **283**, 593-596 (1980).
6. Gray, D. A., Durkacz, B. W. & Shall, S. *FEBS Lett.* **131**, 173-177 (1981).
7. Creissen, D. & Shall, S. *Nature* **296**, 271-272 (1982).
8. Caplan, A. I. & Rosenberg, M. J. *Proc. natn. Acad. Sci. U.S.A.* **72**, 1852-1857 (1975).
9. Rastl, E. & Swetly, P. J. *biol. Chem.* **253**, 4333-4340 (1978).
10. Bredhorst, R., Klapproth, K. & Hiltz, H. *Cell Differentiation* **9**, 95-103 (1980).
11. Farzaneh, F., Shall, S. & Zalin, R. in *Novel ADP-Ribosylations of Regulatory Enzymes and Proteins* (eds Smulson, M. & Sugimura, T.) 217-225 (Elsevier, Amsterdam, 1980).
12. Farzaneh, F., Shall, S., Brill, D. & Zahlin, R. *Nature* **300**, 362-366 (1982).
13. Ling, N. R. & Kay, J. E. *Lymphocyte Stimulation* 2nd edn (North-Holland, Amsterdam, 1975).
14. Wedner, H. J. & Parker, C. W. *Prog. Allergy* **20**, 195-300 (1976).
15. Crumpton, M. J., Perles, B. & Auger, J. in *International Cell Biology* (eds Brinkley, B. R. & Porter, K. R.) 119-127 (Rockefeller University Press, New York, 1977).
16. Shall, S. J. *Biochem., Tokyo* **77**, 2p (1975).
17. Purnell, M. R. & Whish, W. J. D. *Biochem. J.* **185**, 775-777 (1980).
18. Clark, J. B., Ferris, G. M. & Pinder, S. *Biochim. biophys. Acta* **238**, 82-85 (1971).
19. Cook, P. R. & Brazell, I. A. J. *cell. Sci.* **22**, 287-302 (1976).
20. Cook, P. R., Brazell, I. A. & Jost, E. J. *cell. Sci.* **22**, 303-324 (1976).
21. Durkacz, B. W., Irwin, J. & Shall, S. *Eur. J. Biochem.* **121**, 65-69 (1981).
22. Cook, P. R. & Brazell, I. A. *Nature* **263**, 679-682 (1976).
23. Vitti, P., De Wolf, M. J. S., Acquaviva, A. M., Epstein, M. & Kohn, L. D. *Proc. natn. Acad. Sci. U.S.A.* **79**, 1525-1529 (1982).
24. Calabretta, B., Robberson, D. L., Barrera-Saldana, H. A., Lambrou, T. P. & Saunders, G. F. *Nature* **296**, 219-225 (1982).
25. Weisbrod, S. *Nature* **297**, 289-295 (1982).
26. Cook, P. R. *Nature* **245**, 23-25 (1973).
27. MacDermott, A. *New Scientist* **95**, 228-231 (1982).
28. Johnstone, A. P., DuBois, J. H. & Crumpton, M. J. *Biochem. J.* **194**, 309-318 (1981).

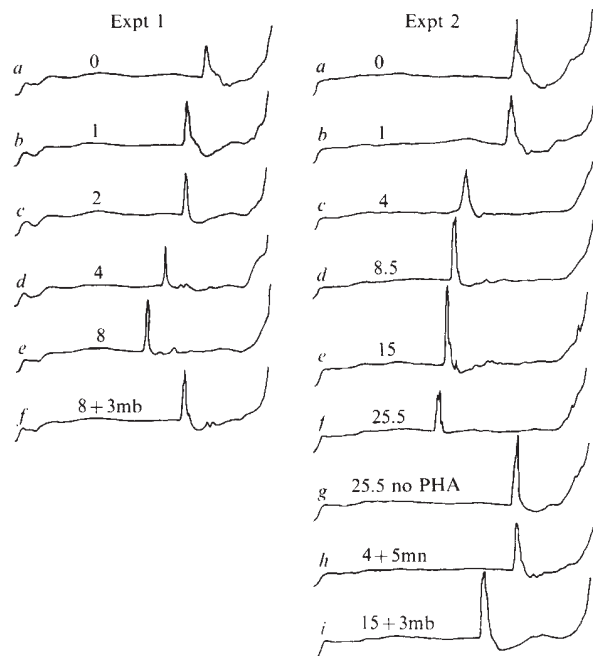


Fig. 3 Changes in nucleoid sedimentation rate during lymphocyte activation. In two separate experiments, lymphocytes were isolated from two individuals and 5×10^5 cells cultured with PHA as described for Fig. 1b. After varying times, the cells were washed once with ice-cold 10 mM sodium phosphate, 0.15 M NaCl pH 7.3 and deposited (in $\sim 100 \mu\text{l}$) inside a layer of 400 μl of 0.5% Triton X-100, 10 mM Tris, 10 mM EDTA, 2 M NaCl pH 8.0 lying on top of a 11.5 ml gradient of 5-20% (w/v) sucrose in 10 mM Tris, 10 mM EDTA, 2 M NaCl pH 8.0 in a Beckman SW41 centrifuge tube. After incubation for 15 min at 20 °C, the gradients were centrifuged at 25,000 r.p.m. (100,000 g_{max}) for 30 min at 20 °C and eluted from the bottom through the flow cell of a Unicam SP8000 recording spectrophotometer set at 260 nm. The absorbance profiles (bottom of gradient on the left) obtained from the following cultures are presented. Expt 1: lymphocytes incubated at 37 °C with PHA for: a, 0; b, 1 h; c, 2 h; d, 4 h; e, 8 h. Before addition of mitogen, cells were incubated for 16 h at room temperature in medium alone (e) or 2.5 mM 3-methoxybenzamide (f). Expt 2: lymphocytes incubated at 37 °C for: a, 0; b, 1 h; c, 4 h; d, 8.5 h; e, 15 h; f, 25.5 h; g, 4 h; h, 4 h; i, 15 h with PHA or for 25.5 h without PHA (g). Before addition of mitogen, cells were incubated for 4 h at 37 °C in medium alone (c and e) or 5 mM 5-methylnicotinamide (h) or 2.5 mM 3-methoxybenzamide (i). Each nucleoid does not sediment totally independently and so the peaks obtained represent the average migration of a heterogeneous population.

A new wild-derived *H-2* haplotype enhancing *K-IA* recombination

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It is generally accepted that the overall recombination frequency between the *H-2K* and *H-2D* loci of the mouse major histocompatibility complex (*H-2*) is at a low level of about 0.4%. However, the value fluctuates depending upon the combinations of *H-2* haplotypes in the heterozygote¹. We re-examined the intra-*H-2* recombination frequency, using three B10.W congenic strains which carry the *H-2* complex of Japanese wild mice. Of the three, one strain, B10.MOL-SGR(*H-2*^{wm7}), showed an extremely high incidence of recombination (about 3%) in all combinations with three different inbred B10 congenic strains. The serological typing of these recombinants revealed that most of the crossing-over points had occurred between the *H-2K* and *IA* loci. These results suggest that the *wm7* haplotype enhances specific recombination irrespective of *H-2* partners in heterozygous parents.

In the past five years we have produced several new *H-2* congenic strains by introducing the *H-2* complex of Japanese wild mice into the C57BL/10J (B10) genetic background². Japanese wild mice, *Mus musculus molossinus*, are genetically very remote from the European subspecies, *Mus musculus domesticus*. It is estimated that ancestral mice diverged into these subspecies about one million years ago^{3,4}. Consequently, many genetic mutations seem to have accumulated in their genomes. Recent genetic research has shown that most inbred laboratory strains are derived from the European subspecies⁵. This finding prompted us to re-examine the intra-*H-2* recombination frequency in heterozygotes between inbred B10 congenic strains and the B10.W strains produced in our laboratory.

All mice used in the present study are shown in Table 1, together with serological typing of their *H-2K*, *IA*, *IE*, *Ss-Slp* and *H-2D* loci. Table 2 summarizes the recombination

Table 1 Immunological characters of *H-2* complex in the mouse strains used

Strain	<i>H-2</i> haplotype	Presence of antigenic specificities expressed by cytotoxic titres											Origin of <i>H-2</i> complex
		K	K.33	Ia.2	Ia.18	Ia.20	IE (IE ^a)	Ss	Slp	D.4	D.32	D.2	
B10.A	<i>a</i>	80	0	>160	>160	0	40	80	h	a	>160	0	Inbred, A/WySn
B10.BR	<i>k</i>	80	0	>160	>160	0	40	80	l	o	>160	0	Inbred, C57BR/cd
B10	<i>b</i>	0	>160	0	0	>160	0	0	h	o	0	>160	Inbred, C57BL/10J
B10.MOL-TEN1	<i>wm1</i>	0	0	0	0	0	0	0	l	o	0	0	Jap. wild (Sapporo)
B10.MOL-SGR	<i>wm7</i>	0	0	0	0	0	40	0	h	o	0	0	Jap. wild (Fukuoka)
B10.MOL-YNG	<i>wm11</i>	0	0	0	0	0	0	0	h	o	0	0	Jap. wild (Yonaguni Is.)

H-2 alloantisera were produced in our laboratory according to the immunization schedules described in the NIH catalogue⁶. These are D23(anti-K.23), D33(anti-K.33), D4(anti-D.4), D32(anti-D.32) and D2(anti-D.2). Monoclonal anti-Ia antibodies were kindly given by Dr K. Ozato of NIH. These are 26-7-11S(anti-Ia.2), 26-8-16S(anti-Ia.18), 25-9-35S(anti-Ia.20), 14-4-S(anti-Ia.7) and 17-3-3S(anti-IE^a). For the last one, the antigenic specificity detected is unidentified. Ss-Slp typing was performed by Ouchterlony double diffusion analysis using rabbit anti-Ss serum and Slp alloantiserum which were kindly given by Dr S. Sakai of Kanazawa University. The microcytotoxicity test was carried out using the flat-type titration plate method previously described⁷. Titres of *H-2* antisera and Ia antibodies are indicated as the maximum dilution which kills more than 90% and 50% of the cells respectively.

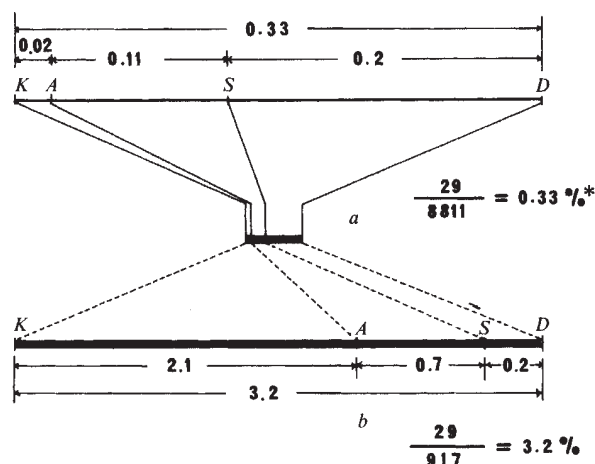


Fig. 1 Comparison between two genetic maps of *H-2* complex. *a*, Standard genetic map previously reported¹. The map distances represent the average recombination frequency (%) in various combinations of *H-2* haplotypes for males and females together. *b*, Map constructed from the data shown in Table 3. The distances between each loci are slightly underestimated, because two recombinants, *aw16* and *kw1*, whose crossing-over origins were unidentified, were excluded from the calculation of distances*. Data from ref. 1.

frequencies estimated from the present experiments. We screened 1,510 mice and detected 30 recombinants. The overall recombination frequency was estimated to be 1.9%, but the values were strikingly different among the combinations of *H-2* haplotypes. Of the five combinations, *a/wm1* and *a/wm11* showed the standard frequency as reported previously by many investigators. On the other hand, the frequency in the heterozygotes between *wm7* and three different inbred *H-2* haplotypes, *a*, *k* and *b*, was 3–4%, which is almost one order of magnitude higher than the normal recombination frequency.

In order to define the positions of crossing-over in the above three *H-2* combinations, the genotypes of the *IA*, *IE* and *Slp* loci were determined by serological typing. Table 3 demonstrates two features of the recombination event involving the *wm7* haplotype. First, the crossing-over positions are concentrated in the chromosomal segment between *H-2K* and *IA* loci, a segment which many laboratories have observed to have the lowest incidence of recombination (0.02%)¹. This is conspicuous in the *k/wm7* and *b/wm7* heterozygotes. Of the 27 recombinants examined, at least 19 are derived from crossing-over in this segment. It should be noted that the average recombination frequency among the three combinations is 2.1%. On the other hand, crossing-over between the *Slp* and *D* loci is rare. Crossing-over between these segments was found in only two recombinants, although this is the site with the

highest incidence of crossing over that has been observed in combinations of inbred *H-2* haplotypes so far. This site-specific enhancement of recombination can be clearly demonstrated by comparing the genetic map constructed from the present data with the standard map. Figure 1 shows that the relative distance between each marker locus becomes larger toward *H-2K* end in the present map, compared with the standard map. Second, reciprocal recombinant types do not seem to occur with equal frequency. In the eight *b/wm7* heterozygotes, only *K^bD^w* recombinants were detected except for *bw7*. This tendency is also shown in the *a/wm7* heterozygote, although the *K^wD^a* type is dominant in this case.

Some of these recombinants were crossed with a third party mouse and the offspring serologically typed with *H-2* antisera. All recombinants examined were confirmed by the test (Table 3). Furthermore, fourteen recombinants derived from the *a/wm7* heterozygote have been established as *H-2* recombinant strains and are now maintained by sib-mating. These were again serologically typed, this time using anti-*H-2^{wm7}* antiserum. For this purpose, (B10.BR × DBA/2J) anti-B10.MOL-SGR alloantiserum was produced and subsequently absorbed by B10.A(R201) (*H-2^{aw1}*, *K^kA^wE^wS^wD^w*) and B10.A(R217) (*H-2^{aw17}*, *K^wA^wE^wS^dD^d*) respectively. It is expected that the resultant two antisera will detect either *H-2D* or *H-2K* specific antigens of the *wm7* haplotype. The reaction of these two antisera to the established recombinant strains was consistent with that expected from the genotypes determined by the antisera detecting the inbred *H-2* haplotypes (Table 3), indicating that genetic rearrangement had occurred in these strains.

This is the first report describing a genetic factor that enhances intra-*H-2* recombination. The mechanism by which the B10.MOL-SGR strain raises the recombination frequency in combinations with other strains is, at present, not clear. However, as all the mice used in the present cross experiment carry the common genetic background of the B10 mouse, the

Table 2 Frequency of intra-*H-2* recombination

<i>H-2</i> haplotype of heterozygous parents	Method of screening	No. of mice screened	No. of recombinants	Recombination frequency (%)
<i>a/wm1</i>	HA*	294	1	0.3
<i>a/wm11</i>	HA	299	0	—
<i>a/wm7</i>	HA	539	16	3.0
<i>k/wm7</i>	CT†	174	5	2.9
<i>b/wm7</i>	CT	204	8	3.9
Total		1,510	30	1.9

To detect the recombinants, the females of *F₁* hybrids between inbred B10 congenic strains and three B10.W strains were backcrossed to the respective parental B10.W strains. The offspring were screened with two *H-2* alloantisera which detect either the *H-2K* or *H-2D* private antigens carried by the parental inbred *H-2* haplotypes. For the typing, haemagglutination test was used if the antiserum was suitable, if not microcytotoxicity tests against inguinal lymph node cells were carried out. * Haemagglutination test; † cytotoxicity test.

Table 3 Genetic composition of the recombinant *H-2* haplotypes

Parental <i>H-2</i> haplotype	Established strain	Recombinant <i>H-2</i> haplotype	Origin of <i>H-2</i> subregions					Progeny test	Cytotoxic titres anti- <i>H-2</i> ^{wm7} adsorbed with	
			<i>K</i>	<i>A</i>	<i>E</i>	<i>S</i>	<i>D</i>		R201	R217
<i>a/wm7</i>	B10.A(R201)	<i>aw1</i>	<i>k</i>	<i>w</i> *	<i>w</i>	<i>w</i>	<i>w</i>	Yes†	0	4
	B10.A(R202)	<i>aw2</i>	<i>k</i>	<i>k</i>	<i>k</i>	<i>d</i>	<i>w</i>	Yes	0	4
	B10.A(R203)	<i>aw3</i>	<i>k</i>	<i>k</i>	<i>k</i>	<i>w</i>	<i>w</i>	Yes	0	4
	B10.A(R204)	<i>aw4</i>	<i>w</i>	<i>k</i>	<i>k</i>	<i>d</i>	<i>d</i>	Yes	64	0
	B10.A(R205)	<i>aw5</i>	<i>w</i>	<i>w</i>	<i>w</i>	<i>d</i>	<i>d</i>	Yes	64	0
	B10.A(R206)	<i>aw6</i>	<i>w</i>	<i>k</i>	<i>k</i>	<i>d</i>	<i>d</i>	Yes	64	0
	B10.A(R207)	<i>aw7</i>	<i>w</i>	<i>k</i>	<i>k</i>	<i>d</i>	<i>d</i>	Yes	64	0
	B10.A(R208)	<i>aw8</i>	<i>k</i>	<i>k</i>	<i>k</i>	<i>d</i>	<i>w</i>	Yes	0	4
	B10.A(R209)	<i>aw9</i>	<i>w</i>	<i>k</i>	<i>k</i>	<i>d</i>	<i>d</i>	Yes	64	0
	B10.A(R211)	<i>aw11</i>	<i>k</i>	<i>k</i>	<i>k</i>	<i>w</i>	<i>w</i>	Yes	0	4
	B10.A(R212)	<i>aw12</i>	<i>w</i>	<i>w</i>	<i>w</i>	<i>d</i>	<i>d</i>	Yes	64	0
	B10.A(R213)	<i>aw13</i>	<i>w</i>	<i>w</i>	<i>w</i>	<i>d</i>	<i>d</i>	Yes	32	0
	B10.A(R214)	<i>aw14</i>	<i>w</i>	<i>k</i>	<i>k</i>	<i>d</i>	<i>d</i>	Yes	64	0
		<i>aw15</i>	<i>w</i>	<i>k</i>	<i>k</i>	<i>d</i>	<i>d</i>	NT‡	NT	NT
		<i>aw16</i>	<i>k</i>	?	?	?	<i>w</i>	NT	NT	NT
	B10.A(R217)	<i>aw17</i>	<i>w</i>	<i>w</i>	<i>w</i>	<i>d</i>	<i>d</i>	Yes	64	0
<i>k/wm7</i>		<i>kw1</i>	<i>k</i>	?	?	?	<i>w</i>	NT		
		<i>kw2</i>	<i>w</i>	<i>k</i>	<i>k</i>	?	<i>k</i>	Yes		
		<i>kw3</i>	<i>w</i>	<i>k</i>	<i>k</i>	?	<i>k</i>	Yes		
		<i>kw4</i>	<i>w</i>	<i>k</i>	<i>k</i>	?	<i>k</i>	Yes		
		<i>kw5</i>	<i>k</i>	<i>w</i>	<i>w</i>	?	<i>w</i>	Yes		
<i>b/wm7</i>		<i>bw1</i>	<i>b</i>	<i>w</i>	<i>w</i>	?	<i>w</i>	Yes		
		<i>bw2</i>	<i>b</i>	<i>w</i>	?	?	<i>w</i>	NT		
		<i>bw3</i>	<i>b</i>	<i>w</i>	<i>w</i>	?	<i>w</i>	Yes		
		<i>bw4</i>	<i>b</i>	<i>w</i>	?	?	<i>w</i>	NT		
		<i>bw5</i>	<i>b</i>	<i>w</i>	<i>w</i>	?	<i>w</i>	Yes		
		<i>bw6</i>	<i>b</i>	<i>w</i>	<i>w</i>	?	<i>w</i>	Yes		
		<i>bw7</i>	<i>w</i>	<i>b</i>	<i>b</i>	?	<i>b</i>	Yes		
		<i>bw8</i>	<i>b</i>	<i>w</i>	<i>w</i>	?	<i>w</i>	Yes		

Antisera and antibodies used to define the origin of *H-2* subregions are shown in Table 1. The *S* region is serologically typed with Slp alloantiserum. Solid vertical bars indicate the crossing-over positions.

* The letter *w* indicates wild-derived subregions. † Tested. ‡ Not tested.

difference in the recombination frequency seems to be a direct result of the *H-2* complex. It seems likely that the chromosomal structure of the *H-2K* end, especially between *H-2K* and *IA*, of the *wm7* haplotype is different from that of other haplotypes. The ultimate interpretation should be made through investigations on the DNA level.

Generally, the intra-*H-2* recombination frequency is higher in the female than in the male. The total frequency in the female was 0.32%, and in the male, 0.19%, based on the progeny-tested recombinants¹. Therefore, the frequency of female recombination estimated in the present experiment is still one order greater than the normal value. We are now proceeding to evaluate male recombination frequency in heterozygotes, between the *wm7* and several other *H-2* haplotypes.

The B10.MOL-SGR strain will provide a powerful tool for investigating the structure and functions of *H-2* genes, because we can obtain the recombinant derived from *K*-end specific crossing-over by screening at most 30–40 mice, probably irrespective of *H-2* haplotypes. Moreover, the resultant recombinants would be useful in detecting unknown Class I and Class II loci if they are located at the *H-2K* end, especially between the *H-2K* and *IA* loci.

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Allelic polymorphism and complexity of the genes for HLA-DR β -chains—direct analysis by DNA–DNA hybridization

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HLA-DR antigens are highly polymorphic cell surface glycoproteins involved in the control of the immune response. They are important in allograft rejection and in their linkage to certain disease susceptibilities. The allelic polymorphism of HLA-DR antigens is carried by the β -chain. We have isolated cDNA clones encoding several distinct HLA-DR β chains and other β -chains from related loci, including DC, making it possible to analyse this complex genetic system directly by Southern hybridization and to demonstrate the polymorphism of these DNA sequences in the human genome. Hybridization patterns obtained with DNA of one DR-heterozygous and three DR-homozygous cell lines suggest the presence of a number of different genes for these two β -chain loci. Extensive polymorphism of the restriction pattern was detected in individuals of different DR specificities, with each of the three restriction enzymes used. Analysis of HLA-DR directly at the DNA level may lead to a simpler typing procedure and may eventually refine the specificities so far determined either serologically or by cellular assay.

The I region of the mouse major histocompatibility complex (*H-2*) controls the immune response¹. These genes encode polymorphic glycoproteins (Ia antigens) expressed mainly at the cell surface of macrophages and lymphocytes and involve

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1. Klein, J. *The Biology of the Mouse Histocompatibility-2 Complex* (Springer, New York, 1975).
2. Moriawaki, K. et al. *Teratocarcinoma and Embryonic Cell Interactions* (eds Muramatsu, T., Gachelin, G., Moscona, A. A. & Ikawa, Y.) 157–175 (Academic, Tokyo, 1982).
3. Moriawaki, K. et al. *J. Immunogenet.* **6**, 99–113 (1979).
4. Yonekawa, H. et al. *Genetics* **98**, 801–816 (1981).
5. Yonekawa, H. et al. *Jap. J. Genet.* **55**, 289–296 (1980).
6. Snell, G. D. in *Catalog of Mouse Alloantisera* (National Institutes of Health, Bethesda, 1968).
7. Shiroishi, T., Sagai, T. & Moriawaki, K. *Microbiol. Immun.* **25**, 1327–1334 (1981).

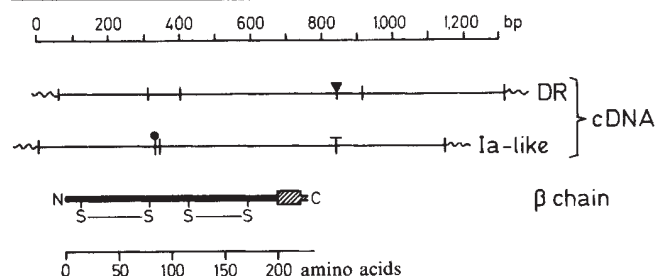


Fig. 1 Maps of cDNA clones encoding two distinct human Ia antigen β -chains. Clones were isolated as described elsewhere¹⁴. Clone DR β -1 encodes a β -chain of HLA-DR antigen. Clone Ia-like β -1 encodes a β -chain of another related antigen, probably DC. The sites for several restriction endonucleases are indicated by the following symbols: *Pst*I ($\downarrow$), *Hind*III (∇), *Bam*HI ($\uparrow$), and *Eco*RI ($\vdash$). Wavy lines represent pBR322 sequences. The diagram of the β -chain structure is based on the sequence of a human Ia antigen β -chain determined by Kratzin *et al.*²³. The transmembrane region, shown by a hatched box, and the cytoplasmic region have not been sequenced.

cellular interactions essential for the immune response. The equivalent antigens in humans are coded for by the HLA-D region of the major histocompatibility complex^{2,3}. The best known, HLA-DR antigens, consist of an invariant heavy or α -chain² and a highly polymorphic light or β -chain^{4,6}. Monoclonal antibodies have distinguished subsets of DR antigens⁷. Typing of the polymorphic DR specificities, important for successful organ transplantation⁸, is done by serological tests and by the mixed lymphocyte reaction⁹. In addition, there is significant linkage between some HLA-DR specificities and susceptibility to certain diseases¹⁰. Besides DR, other human Ia antigens have been identified serologically. Their genes (DC) are in close linkage disequilibrium with the classical polymor-

phic DR locus^{11,12}. A third locus, termed SB, has been shown to encode polymorphic Ia antigens¹³.

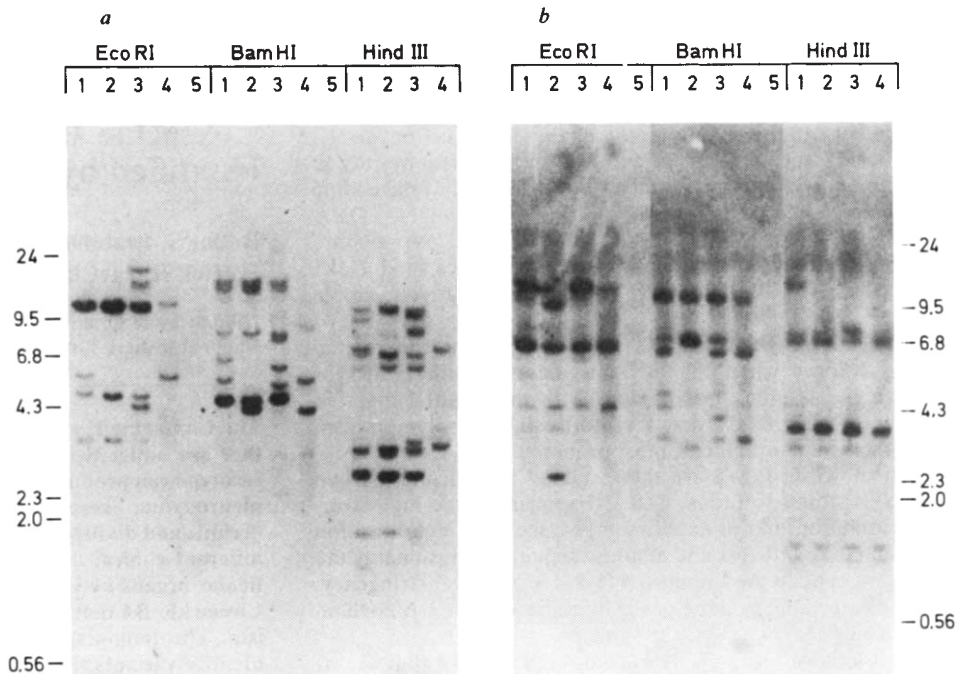
We have cloned cDNA sequences encoding β -chains of HLA-DR antigens and other related Ia-like antigens¹⁴. Initial studies of these clones, and of other recombinant clones containing corresponding DNA sequences from genomic DNA, strongly suggest the existence of several distinct genes for the HLA-DR β chain and for related Ia-like antigen β chain (data not shown). The availability of these β -chain cDNA clones makes possible a direct analysis of this polymorphic genetic system at the level of the DNA. Using the cloned β -chain sequences as probes we have analysed digested human DNA transferred to nitrocellulose filters by the method of Southern¹⁵. First, determining the number of different DNA fragments hybridizing to the β -chain probes will yield information about the complexity of these loci. Second, a comparison of the DNA from individuals with different HLA-DR specificities will quantify the allelic polymorphism of the system.

Restriction enzyme maps of the two β -chain sequences used as hybridization probes are shown in Fig. 1. Both cDNA clones were derived from the lymphoblastoid cell line Raji, which is heterozygous at the DR locus (DR 3, w6). HLA-DR β -1 encodes the DR β chain¹⁴. Ia-like β -1 encodes the β -chain of another human Ia antigen and has been shown to correspond in the mouse to the I-A β chain gene of the I region¹⁶. Since structural studies have now established a correlation between the α -chains of mouse I-A and human DC¹⁷, we can tentatively consider the 'Ia-like' β -1 clone as specific of DC β chain. DNA from one DR-heterozygous and three DR-homozygous human lymphoblastoid cell lines were analysed: Raji (DR 3, w6), HHK (DR w6, w6), IBW9 (DR 7, 7), and LG2 (DR 1, 1).

The results of hybridizing the two β probes to the four genomic DNAs are shown in Fig. 2. A fairly complex pattern is evident. Depending on the cell line and the restriction endonuclease used, 4–13 bands hybridize with the HLA-DR

Fig. 2 Hybridization of cDNA

sequences of the β -chains of HLA-DR and another Ia-like locus to human and mouse DNA. ³²P-labelled probes were purified inserts from cDNA clones HLA-DR β -1 (panel a) or Ia-like β -1 (panel b). DNA samples were from: 1, human B cell line Raji (DR 3, w6); 2, human B cell line HHK (DR w6, w6); 3, human B cell line IBW9 (DR 7, 7); 4, human B cell line LG2 (DR 1, 1); and 5, BALB/c mouse embryo (H-2^b). Size markers are given in kilobases. High molecular weight DNA was isolated as described elsewhere¹⁹. Restriction endonuclease digestions were done at 37 °C with *Eco*RI (Boehringer Mannheim), *Bam*HI or *Hind*III (Bethesda Research Laboratories) using standard buffer conditions with 1 unit enzyme μ g⁻¹ for 4 h, at which time another 1 unit enzyme μ g⁻¹ was added and incubated overnight. Reactions were stopped with EDTA, extracted once with chloroform/isoamyl alcohol (24 : 1), and ethanol-precipitated. Pellets were resuspended in 10 mM Tris-HCl, pH 7.6, 1 mM EDTA, 0.1% SDS, 0.05% bromophenol blue, 0.05% xylene cyanol, and 5% glycerol for 4 h at 37 °C. Samples were heated for 5 min at 65 °C just before loading on to 0.6% agarose gels in 200 mM glycine, 15 mM NaOH, pH 8.3. About 7 μ g of LG2 DNA and 9 μ g of the other DNA samples were loaded. Gels were run at 60–100 V for 12 h, treated and transferred to 0.2 μ m nitrocellulose filters (Schleicher and Schuell) as described²⁴. After transfer, the filters were rinsed in 4 $\times$ SSC (SSC = 150 mM NaCl, 15 mM trisodium citrate), then baked for 2 h at 80 °C in a vacuum oven. Filters were prewashed in 5 $\times$ SSC, 5 $\times$ Denhardt's solution for 1–2 h at 65 °C with gentle shaking, then prehybridized for 2 h at 65 °C in 1 $\times$ Denhardt's, 0.75 M NaCl, 5 mM EDTA, 50 mM sodium phosphate buffer, pH 7.0, 10% dextran sulphate, 0.1% SDS, and 50 μ g ml⁻¹ sonicated denatured herring DNA. Hybridization was for 8–12 h at 65 °C in the same buffer as used for prehybridization with 1 $\times$ 10⁶ c.p.m. ml⁻¹ of ³²P-labelled DNA (>10⁸ c.p.m. μ g⁻¹). Filters were washed at 65 °C for 30 min twice with each of the following solutions: 5 $\times$ SSC, 1 $\times$ Denhardt's, 0.1% SDS, 0.1% sodium pyrophosphate; 2 $\times$ SSC, 0.1% SDS; 0.5 $\times$ SSC; 0.1 $\times$ SSC.



β -1 clone and 3-9 bands hybridize with the Ia-like β -1 clone. This multiplicity of bands contrasts markedly with the simple pattern obtained when the cDNA clone encoding the non-polymorphic HLA-DR α -chain is used as probe^{18,19}. However, the degree of complexity of the HLA-DR β chain sequences observed here contrasts also with the more extensive complexity found in class I genes of both man²⁰ and mouse^{21,22}, where up to 40 DNA fragments hybridize to the specific HLA or H-2 probes.

The finding of multiple hybridizing bands is a strong indication of the existence of several genes for the β -chains of both HLA-DR and the Ia-like (or DC) loci. This is in complete agreement with our recent finding in genomic and cDNA clones from homozygous cell lines of several distinct genes for both the HLA-DR β -chain and the β -chain of the DC locus, as well as several additional genes for other related Ia-like β chains, not yet identified (data not shown). Some of the weakly hybridizing bands could result from sequence differences between the probe (isolated from Raji cells) and the homologous sequences in other cell lines. The existence of strongly cross-hybridizing bands in each of the cell lines indicates highly conserved regions in the β -chain gene. In addition, weak signals can also result from DNA fragments containing only a short region of homology.

The genetic polymorphism of the HLA-DR locus, which previously could be demonstrated and assayed only at the level of the expressed proteins, is detected here directly at the level of the DNA. The four cell lines with different DR specificities generated different patterns of hybridizing bands with all three restriction endonucleases tested (Fig. 2a). For example, with *Bam*HI digestion, only one weak band is shared by all four cell lines out of their 4–11 bands. Only a few of these 4–11 fragments are unique to any one cell line. This analysis of genomic DNA can detect not only polymorphic endonuclease restriction sites located within the coding region, and corresponding to polymorphic changes seen in the antigens, but also possible polymorphic sites located within non-coding introns or in sequences flanking the DR genes. The *Eco*RI, *Hind*III and *Bam*HI restriction sites present in the coding regions will probably not account for all of the observed polymorphism. This indicates that the polymorphism observed with an HLA-DR β -chain probe extends outside the coding region of these genes. Conversely, by choosing specific probes and specific restriction sites, it should be possible to focus and limit the analysis to the coding region concerned in the polymorphism of the antigens. DNA from the DR-heterozygous line Raji does not give a more complex pattern than the homozygous cell lines, and DNA from a HLA-DR 1,1 homozygous shows a relatively simple pattern.

Significant polymorphism is also observed at the related Ia-like β locus when the DNA is digested with *Eco*RI or *Bam*HI, but is much less obvious with the *Hind*III-digested samples (Fig. 2b). These data therefore establish polymorphism at another human Ia locus, most probably HLA-DC.

Also shown in Fig. 2 are the results of hybridizing the two human β probes to mouse DNA. Even under the high stringency conditions of this experiment, we see cross-hybridization between HLA-DR β -1 and a mouse sequence, presumably the analogous gene in the I region of H-2. Under lower stringency, the Ia-like (or DC) β -chain probe hybridizes to the I-A β -chain in mouse DNA¹⁶.

The Southern blot and hybridization procedure thus seems to be a simple and precise technique for detecting HLA-DR allelic polymorphism directly at the DNA level. A more extensive investigation would be necessary to correlate the DR specificities determined classically by serological and cellular tests with the hybridization pattern of the DNA. Such a study would test the feasibility and reliability of 'DNA typing' for DR specificity. In addition, Southern blot analyses might reveal subtle differences in the DNA patterns which could allow a further subdivision of the known DR specificities. Increased precision and large-scale availability of HLA-DR typing would

be important because of the association between particular HLA-DR specificities and susceptibility to certain diseases.

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1. Klein, J. *Science* **203**, 516–521 (1979).
2. Strominger, J. L. et al. in *The Role of the Major Histocompatibility Complex in Immunobiology* (ed. Dorf, M. E.) 115–172 (Garland, New York, 1981).
3. Bodmer, W. F. *Tissue Antigens* **17**, 19–20 (1981).
4. Silver, J. & Ferrone, S. *Nature* **279**, 436–437 (1979).
5. Charron, D. J. & McDavitt, H. O. *J. exp. Med.* **152**, 188–366 (1980).
6. Shackelford, D. A. & Strominger, J. L. *J. exp. Med.* **151**, 144–165 (1980).
7. Accolla, R. S., Gross, N., Carrel, S. & Corte, G. *Proc. natn. Acad. Sci. U.S.A.* **78**, 4549–4551 (1981).
8. Dausset, J. *Science* **213**, 1469–1474 (1981).
9. Bach, F. H. & van Rood, J. J. N. *Engl. J. Med.* **295**, 806–813 (1976).
10. Ryder, L. P., Sveigaard, A. & Dausset, J. A. *Rev. Genet.* **15**, 169–187 (1981).
11. Corte, G., Calabi, F., Damiani, G., Bargellesi, A., Tosi, R. & Sorrentino, R. *Nature* **292**, 357–360 (1981).
12. Shackelford, D. A., Mann, D. L., van Rood, J. J., Ferrara, G. B. & Strominger, J. L. *Proc. natn. Acad. Sci. U.S.A.* **78**, 4566–4570 (1981).
13. Shaw, S. et al. *J. Exp. Med.* **156**, 731–741 (1982).
14. Long, E. O. et al. *Proc. natn. Acad. Sci. U.S.A.* **79** (in the press).
15. Southern, E. M. *J. molec. Biol.* **98**, 503–517 (1975).
16. Steinmetz, M. et al. *Nature* (in the press).
17. Bona, M. R. & Strominger, J. L. *Nature* **299**, 836–838 (1982).
18. Lee, J. S., Trowsdale, J. & Bodmer, W. F. *Proc. natn. Acad. Sci. U.S.A.* **79**, 545–549 (1982).
19. Wake, C. T. et al. *Proc. natn. Acad. Sci. U.S.A.* **79**, 6979–6983 (1982).
20. Orr, H. T. et al. *Nature* **296**, 454–456 (1982).
21. Cami, B., Brégère, F., Abastado, J. P. & Kourilsky, P. *Nature* **291**, 673–673 (1981).
22. Steinmetz, M. et al. *Cell* **24**, 125–134 (1981).
23. Kratzin, H. et al. *Hoppe-Seyler's Z. physiol. Chem.* **362**, 1665–1669 (1981).
24. Wahl, G. M., Stern, M. & Stark, G. R. *Proc. natn. Acad. Sci. U.S.A.* **76**, 3683–3687 (1979).

High frequency of antigenic variants among naturally occurring human Coxsackie B4 virus isolates identified by monoclonal antibodies

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The Coxsackie B virus group consists of six serotypes (B1–B6) that are antigenically distinct. In patients, the Coxsackie B4 serotype can produce a variety of clinical diseases (for example, pleurodynia, respiratory illness, meningitis, myocarditis, orchitis and diabetes)^{1,2}. It is not known, however, whether the different clinical diseases are due to chance infection of particular organs by Coxsackie B4 or due to antigenic variants of Coxsackie B4 that are inherently different in their tissue tropism. The long-standing reference hyperimmune sera do not identify variants that may exist within each of the serotypes³. In an attempt to identify variants of the Coxsackie B4 serotype, we prepared a panel of 18 monoclonal antibodies that neutralized the prototype Coxsackie B4 strain. These monoclonal antibodies have been characterized as to their subclass, neutralization titres and reactivity patterns with recent clinical isolates. Here we have identified over 13 variants of Coxsackie B4 virus and have shown that there are major antigenic differences among naturally occurring isolates. Using monoclonal antibodies as a selecting agent, we find that the frequency of antigenic mutation may be as high as 10⁻⁴.

Antibodies	Isolates of Coxsackie B4															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
HI*																
86-3																
204-4																
183-1																
286-1																
287-3																
225-1																
9-4																
204-3																
356-1																
187-1																
38-1																
215-2																
339-1																
337-3																
302-1																
128-2																
208-2																
288-3																
59-3																
17-1																

Fig. 1 Neutralization of clinical isolates by monoclonal antibodies. 100 TCID₅₀ of each of the 15 clinical isolates (numbered 2 to 16) and the prototype virus (1) were incubated with each of the monoclonal antibodies and the hyperimmune sera (HI) in a microneutralization assay (see legend to Table 1). The entire experiment was repeated twice with comparable results. Open boxes indicate complete neutralization of virus; hatched boxes indicate that the virus was not neutralized.

Hybridomas which secrete monoclonal antibodies specific for Coxsackie B4 virus were produced by fusing P₃ 653 Ag8 myeloma cells with spleen cells from BALB/c mice that were injected five times intraperitoneally with 10⁷ plaque-forming units (PFU) of purified prototype Coxsackie B4 virus (JVB strain) obtained from the American Type Culture Collection (ATCC VR-184). The fusion protocol and selection of clones were as described previously^{4,5}. Initially, the hybridomas were selected by screening the culture supernatants for virus-specific antibody in a microneutralization assay against the prototype virus. Cultures that were positive for antibody were cloned using the end point dilution method⁶. Clones grown from a single cell were further tested in a radioimmunoassay (RIA) for the production of antibody.

More than 70 monoclonal antibodies arising from 22 hybridomas that neutralize the prototype Coxsackie B4 virus have been obtained. The properties of 18 of these monoclonal antibodies are shown in Table 1. The monoclonal antibodies were predominantly of the IgG class and exhibited different neutralization titres against the prototype virus and precipitated different amounts of virus by RIA. For example, antibody 225-1 had a neutralization titre of 1,536 but bound only 10.8% of the virus in RIA. In contrast, antibody 38-1 had a neutralization titre of only 96, but bound 100% of the virus in RIA. These differences between RIA and neutralization could be due either to varying capacities of the antibody subclasses to bind to the *Staphylococcus aureus* cells used in the RIA⁷ or to different concentrations of various epitopes required for neutralization. Monoclonal antibody 59-3 failed to neutralize the prototype virus and together with 17-1 served as a control. Most of the hybridomas have been grown in BALB/c mice⁸, and ascites fluid with neutralization titres greater than 1:130,000 have been obtained. The monoclonal antibodies in Table 1 were also tested by neutralization against other serotypes of the Coxsackie B virus group (B1, B2, B3, B5 and B6) and found to be non-reactive.

The 18 neutralizing antibodies described in Table 1 were used for the antigenic analysis of 15 fresh clinical isolates obtained from two different State Health Departments. Each isolate was passaged three times or less in culture. Although all the isolates were neutralized by type-specific (anti-B4) hyperimmune reference serum, the monoclonal antibodies show that there is considerable antigenic variation among the Coxsackie B4 isolates (Fig. 1). Some of the monoclonal antibodies (that is 86-3, 204-4) neutralized all the isolates, whereas others (128-2, 208-2, 288-3) neutralized none of the isolates. Figure 1 also shows that the monoclonal antibodies recognized 15 different epitopes. The clinical isolates differed from the prototype virus in 2–12 of the 15 different epitopes. Some of the isolates (for example, 11 to 13) appear to be antigenically identical, others differ from each other at only one epitope (for example, 2 and 3; 6 and 7; 8 and 9), and still others differ from each by as many as 10 epitopes (for example, 2 and 16). Analysis of the data revealed 13 different antigenic variants. Although there were some exceptions (for example, 286-1), the monoclonal antibodies that gave the highest neutralization titres against prototype virus (Table 1), usually neutralized the greatest number of clinical isolates (Fig. 1). One possible explanation for such a broad specificity is that the epitopes with which these monoclonal antibodies are reacting are present in high concentration on the surface of prototype virus and are broadly expressed among the clinical isolates.

The identification of a large number of naturally occurring clinical variants by monoclonal antibodies suggested that the rate of mutation of Coxsackie B4 virus might be high. To test

Table 1 Reactivity of monoclonal antibodies with prototype Coxsackie B4 virus

Antibodies	Subclass	RIA (% bound)	Neutralization titre against prototype virus
Hyperimmune serum	—	100.0	1,024
86-3	IgG2b	42.0	4,056
204-4	IgG2a	75.9	192
183-1	IgG1	100.0	768
286-1	IgG2b	91.9	12
287-3	IgG2a	82.2	1,536
225-1	IgG2b	10.8	1,536
9-4	IgG3	51.5	384
204-3	IgG*	88.1	24
356-1	IgG2a	69.3	96
187-1	IgG2a	87.8	48
38-1	IgG2a	100.0	96
215-2	IgG2a	92.3	48
339-1	IgG2a	86.9	48
337-3	IgG2a	58.9	24
302-1	—†	97.1	12
128-2	IgM	61.9	6
208-2	IgG2b	7.2	2
288-3	—†	13.0	6
59-3	—†	27.0	<2
17-1	—†	0.9	<2

Prototype virus and clinical isolates were grown in LLC-MK2 (MK) cells to a maximum titre of 2×10^6 TCID₅₀ ml⁻¹ (Tissue Culture Infective Dose calculated by the Reed-Muench method¹⁷). These viruses were diluted to contain 100 TCID₅₀ in 0.1 ml before being used in the microneutralization test. The monoclonal antibodies were from tissue culture supernatants. The subclass was determined by double diffusion using rabbit anti-mouse IgG1, IgG2a, IgG2b, IgG3 antisera (Miles Laboratories), and goat anti-mouse IgG (heavy chain) and IgM (heavy chain) (Cappel Laboratories). For the RIA, monoclonal antibodies (50 µl) were mixed with ³H-leucine-labelled purified Coxsackie B4 virus¹⁶ (1,500 d.p.m.) in 150 µl of buffer (0.01 M Tris, 0.1 M NaCl, 0.001 M EDTA, 1 mg ml⁻¹ bovine serum albumin and 0.01% Triton X-100). After overnight incubation at room temperature, the immune complexes were precipitated using heat-killed, formalin-fixed *S. aureus* cells⁷. Both the supernatant and the precipitate were counted in a liquid scintillation counter and the per cent radiolabelled virus bound by antibody was calculated. To determine the neutralization titre, 100 TCID₅₀ of virus in 50 µl were added to wells of a microtitre plate containing 50 µl of threefold serially diluted monoclonal antibodies. These were mixed and incubated at 37°C for 1 h and then 10⁴ MK cells in 0.1 ml of growth medium were added to each well. The plates were examined for virus-induced cytopathology 7 days later. All samples were tested in duplicate in two separate experiments with similar results. Titres are expressed as the reciprocal of the highest dilution of antibody preventing cytopathology.

* Subclass not determined.

† No precipitin line noted.

Table 2 Frequency of antigenic variants in plaque-purified Coxsackie B4 viruses

Virus	None*	86-4			Monoclonal antibodies 102-2		183-5		204-4	
		Titre	Titre	Freq.	Titre	Freq.	Titre	Freq.	Titre	Freq.
1-1	6.76	1.60	—5.16	1.48	—5.28	1.79	—4.97	2.00	—4.76	
5-2	6.78	1.00	—5.78	ND		ND		1.90	—4.88	
9-2	6.58	1.78	—4.80	1.30	—5.28	1.00	—5.58	2.57	—4.01	
14-2	6.48	1.84	—4.64	1.00	—5.48	ND		1.78	—4.70	
15-4	6.98	2.78	—4.20	2.48	—4.50	2.04	—4.94	2.28	—4.70	

The prototype virus (1-1) and four different clinical isolates (5-2, 9-2, 14-2 and 15-4) were plaque-purified twice in MK cells with agarose overlay. The plaque-purified viruses were grown up in MK cell monolayers and virus titres determined. Serial 10-fold dilutions of the virus were incubated at 37 °C for 1 h in the absence or presence of undiluted monoclonal antibodies (ascites fluid with titres >1:130,000). The virus-antibody mixtures were then added to monolayers of MK cells in 60 mm Petri dishes and adsorbed for 90 min. The inoculum was removed and the cultures were overlaid with medium containing the same antibodies used for neutralization plus 5% methylcellulose. This was done to prevent any residual virus from reinfecting cells. After 7 days, cells were stained with neutral red and the number of plaques were counted and the virus titre determined (expressed in log₁₀). The frequency of mutation was determined by dividing the virus titre in the absence of antibody by the virus titre in the presence of antibody. ND, no plaques observed due to complete neutralization of virus even at the highest concentration of virus inoculum.

* Ascites fluid from a hybridoma that does not produce antibody against Coxsackie B4 virus.

this, prototype virus and four plaque-purified clinical isolates were selected and incubated with four different monoclonal antibodies. Table 2 shows that virus escaping neutralization was found in 17 of the 20 combinations, and, based on the titre of the residual virus, the frequency of mutation was calculated to be 10^{-4.0} to 10^{-5.8}. To ensure that the residual virus represented true variants, plaques were selected randomly from each of the 17 combinations, grown up and re-tested in the absence or presence of the selecting monoclonal antibody. In each case, the selecting monoclonal antibody failed to neutralize the virus, indicating that true antibody-resistant variants had been obtained (data not shown).

In the present study, 18 of 70 antibodies were characterized and their interaction with 15 fresh clinical isolates revealed that the Coxsackie B4 serotype consists of many antigenic variants. Further experiments with another 36 clinical isolates revealed additional antigenic variants (unpublished data). Previously, Coxsackie B4 variants went undetected because polyclonal reference sera were used for typing. The demonstration of a large number of naturally occurring variants suggests that the different clinical pictures seen in Coxsackie B4 infection might be due to variants having different tissue tropism. There are, in fact, specific receptors for picornaviruses on the surface of cells⁹⁻¹¹. If the antigenic determinants in question are involved in virus attachment to the cell receptor, this could affect the binding affinity of the virus and influence the tropism of the virus for various tissues. Studies are now underway to characterize the antigenic pattern of a large number of Coxsackie B4 isolates obtained from patients with different clinical diseases. If a correlation between antigenic variation and disease pattern could be established, then monoclonal antibodies might prove useful in classifying subtypes of Coxsackie B4 virus. Furthermore, these monoclonal antibodies may be of use in epidemiological studies of antigenic variants of Coxsackie B4 virus obtained in different years and from different geographical locations.

Our studies with monoclonal antibodies as a selecting agent show that Coxsackie B4 mutates at a high rate and that the frequency of antigenic variants may be as great as 10⁻⁴. This is rather higher than that reported for influenza virus¹² but is within the range of several estimates for other RNA viruses¹³⁻¹⁵. The high rate of mutation may account for the surprisingly large number of antigenic variants at Coxsackie B4 found in the human population. The fact that some of the variants differed from prototype virus by as many as 12 epitopes suggests that major antigenic changes may periodically develop and thus account for both the persistence of the virus in nature and the different clinical syndromes.

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- Gear, J. H. S. & Measroch, V. *Prog. med. Virol.* **15**, 42-62 (1973).
- Yoon, J. W., Austin, M., Onodera, T. & Notkins, A. L. *N. Engl. J. Med.* **300**, 1173-1179 (1979).

- Melnick, J. L. & Hampil, R. *WHO Bull.* **33**, 761-772 (1965).
- Koprowski, H., Gerhard, W. & Croce, C. M. *Proc. natn. Acad. Sci. U.S.A.* **74**, 2985-2988 (1977).
- Kohler, G., & Milstein, C. *Nature* **256**, 495-497 (1975).
- McKearn, T. J. in *Monoclonal Antibodies* (eds Kennett, R. H., McKearn, T. J. & Bechtol, K. B.) 374 (Plenum, New York, 1980).
- Goding, J. D. (1978). *J. immun. Meth.* **20**, 241-253.
- McKearn, T. J. in *Monoclonal Antibodies* (eds Kennett, R. H., McKearn, T. J. & Bechtol, K. B.) 403-404 (Plenum, New York, 1980).
- Dimmock, N. J. *J. gen. Virol.* **59**, 1-22 (1982).
- Morishima, T., McClintock, P. R., Aulakh, G. S., Billups, L. C. & Notkins, A. L. *Virology* (in the press).
- Crowell, R. L. in *Cell Membrane Receptors for Viruses, Antigens and Antibodies, Polypeptide Hormones and Small Molecules* (eds Beers, R. F. Jr & Bassett, E. G.) 179-202 (Raven, New York, 1976).
- Gerhard, W., Yewdell, J., Frankel, M. E. & Webster, R. *Nature* **290**, 713-717 (1981).
- Wiktorski, T. J. & Koprowski, H. *J. exp. Med.* **152**, 99-112 (1980).
- Birrer, M. J., Udem, S., Nathanson, S. & Bloom, B. R. *Nature* **293**, 67-69 (1981).
- Holland, J. *et al. Science* **215**, 1577-1585 (1982).
- Rueckert, R. R. & Duesberg, P. H. *J. molec. Biol.* **17**, 490-502 (1966).
- Reed, L. J. & Muench, H. *Am. J. Hyg.* **27**, 493-497 (1938).

Synthesis *in vitro* of an exceptionally long RNA transcript promoted by an *AluI* sequence

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More than 300,000 copies of the ~300 base pair (bp)-long *AluI* DNA sequence are dispersed throughout the human genome¹⁻⁷. Several *AluI* sequences have been shown to contain sequences which promote transcription *in vitro* by the enzyme RNA polymerase III⁸⁻¹¹. While the function(s) of transcripts synthesized from such promoters is unclear, one notion is that RNA polymerase III transcription from an *AluI* promoter might have an effect on expression of a nearby RNA polymerase II gene, specifically, the β -globin gene (see Fig. 1). However, we found that this *AluI* promoter is expressed efficiently *in vitro* only in conditions dramatically different from those required for the β -globin and other promoters (both RNA polymerases II and III). Surprisingly, the major transcript produced from this promoter is 2.3 kilobases (kb) long, much longer than any RNA polymerase III transcript described previously.

The DNA structure in the region around the β -globin gene is shown in Fig. 1. The β -globin promoter has previously been shown to be active in the whole-cell lysate transcription system used here¹², although transcription from the *AluI* promoters was not detected. However, transcription from the distal *AluI* repeat was observed by one group in a system containing only RNA polymerase III activity⁹.

The plasmid pHB8 cleaved with *EcoRI* was used as a template for *in vitro* transcription in an experiment in which the amount of lysate added to reaction mixtures was varied from 60 to 25% of the total reaction volume (Fig. 2a-d). At high lysate concentrations, the predominant transcript is a 1.4-kb run-off RNA which arises from RNA polymerase II transcription originating

from the β -globin promoter¹². As the amount of lysate in the reaction mixture is reduced, this RNA is no longer detected. However, a very abundant RNA, ~510 nucleotides long, is now observed (Fig. 2c, d). As shown below, this RNA results from initiation by RNA polymerase III at the β -globin-distal *AluI* repeat sequence. (Initiation from the other *AluI* repeat has not been detected.)

The specific requirements for efficient transcription by RNA polymerase III observed with this promoter are unusual, and not, for example, common to all genes transcribed by this polymerase. Figure 2e-h shows the results obtained when a cloned fragment of adenovirus serotype 2 (Ad2) DNA containing both an RNA polymerase II and III promoter was transcribed in the same set of conditions used with the globin DNA above. While the RNA polymerase II promoter shows the same response to variations in the amount of lysate added as does the β -globin promoter, the RNA polymerase III promoter is used efficiently at all lysate concentrations tested, with a maximum observed at about 50% lysate (Fig. 2f). We have tested one other RNA polymerase III promoter (rat tRNA Leu, obtained from D. Rodi and B. Haas), and found that this promoter responds to variations in lysate concentration in the same manner as the Ad2 RNA polymerase III promoter (results not shown).

When the concentration of lysate is changed, several parameters are simultaneously varied. To determine which of these are responsible for the decrease in transcription of the 510-nucleotide RNA at high lysate concentrations, the following experiment was done. Transcription was first carried out exactly as shown in Fig. 2d (that is, 24% lysate). When the concentration of lysate buffer was increased so as to equal the amount present in reaction mixtures containing 60% extract, no specific transcription was detected (results not shown). Therefore, it seems unlikely that the high protein concentration in the reaction mixtures containing 60% extract inhibits transcription. Furthermore, increasing the buffer concentration did not result in synthesis of the 1.4-kb β -globin RNA, indicating that a high protein concentration is required for transcription initiation from this promoter, as has been observed for other RNA polymerase II promoters¹³. The concentration of each buffer component was then varied independently. Increasing $MgCl_2$ from 2.4 to 6.0 mM resulted in an approximately sixfold inhibition; increasing KCl from 24 to 60 mM inhibited transcription threefold, and an increase in glycerol concentration from 4 to 10% caused a 1.5-fold inhibition. The basis for this response is unclear, although an effect on the double-helical structure of the DNA template is a possibility. A similar effect on transcription of an *Escherichia coli* tRNA gene by *E. coli* RNA polymerase has been observed previously¹⁴. Whether such stringent ionic conditions are required to obtain efficient transcription *in vitro* from any (or all) other *AluI* promoters is also unknown.

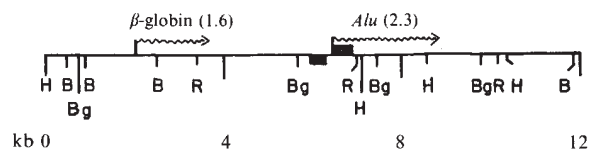


Fig. 1 Map of the human β -globin gene and surrounding sequences. Two inverted *AluI* repeat sequences are indicated by black boxes. The β -globin primary transcript is indicated by a wavy line, as is the *AluI*-generated transcript described here. Sizes are in kb. Relevant restriction enzyme sites are: H, *HindIII*; B, *BamHI*; Bg, *BglII*; R, *EcoRI*. In the experiments described here, we have used primarily two recombinant plasmids as templates for *in vitro* transcription experiments. One, pHB8 (provided by T. Maniatis) contains a 7.5-kb *HindIII* fragment (extending from 0 to 7.5) inserted into pBR322. This fragment contains the entire β -globin gene, as well as two *AluI* repeat sequences lying downstream from it. The other, pHB9.3, contains a 9.3-kb *BamHI* fragment (from about 3 to 12 kb) subcloned into pBR322 from a λ phage recombinant, λ H β G3 (ref. 5) (obtained from T. Maniatis).

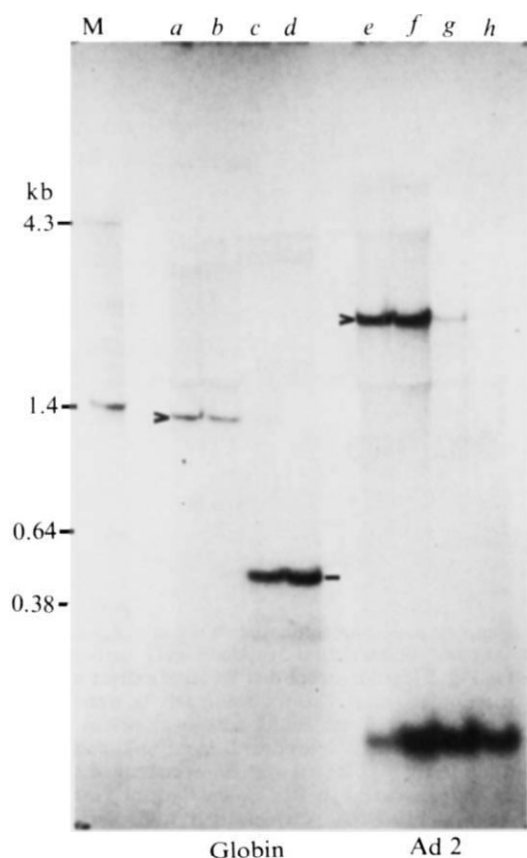


Fig. 2 Response of various promoters to alterations in the concentration of HeLa whole-cell lysate in *in vitro* reaction mixtures. HeLa whole-cell lysates were prepared as described elsewhere^{13,20} except that the final dialysis buffer was 40 mM Tris-HCl pH 7.9, 100 mM KCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 2 mM dithiothreitol, 17% glycerol. The protein concentration of the lysate used in these experiments was ~25 mg ml⁻¹, although similar results have been obtained with less concentrated lysates. Transcription reactions (25 μ l total volume) were carried out as described elsewhere²⁰ except that the concentration of each ribonucleotide triphosphate was 50 μ M, the radioactive precursor was [α -³²P]GTP (5 μ Ci per reaction; NEN), creatine phosphate was present at 4 mM, and the DNA concentration was 90 μ g ml⁻¹. The concentrations of the various buffer components were determined entirely by the amount of lysate added to each reaction mixture. The samples shown in a-d were obtained from reaction mixtures containing pHB8 cleaved with *EcoRI* as template, while the Ad2-derived recombinant pHIIIB*, cleaved with *KpnI*, was the template in the reaction mixtures represented in e-h. The Ad2 fragment in this plasmid extends from 16.4 to 31.5 map units (1 map unit (m.u.) = 360 bp) and contains the RNA polymerase II late promoter (16.5 m.u.), as well as the two small viral-associated (VA) genes (29-30 m.u.), which are transcribed by RNA polymerase III. Cleavage with *KpnI* (23.5 m.u.) leads to the synthesis of a 2.5-kb RNA polymerase II run-off transcript. The RNA polymerase III product, which represents primarily transcription of the VA I gene²¹, is ~160 nucleotides. The amount of lysate added to each reaction mixture was: 15 μ l (lanes a and e), 12 μ l (lanes b and f), 9 μ l (c, g), or 6 μ l (d, h). After incubation at 30 °C for 60 min, RNA was extracted, denatured with glyoxal, and one-quarter of each sample resolved by electrophoresis through a 1.4% agarose gel. The autoradiograph was obtained by exposure of the dried gel to Kodak XAR-5 film for 24 h (without intensifying screens). If quantitation was required, gels were scanned with a Gilford densitometer. RNA polymerase II products are indicated by the arrowheads, RNA polymerase III products by the short lines. The marker (lane M) consists of run-off RNAs obtained from *in vitro* transcription of the plasmid p04, as described previously²².

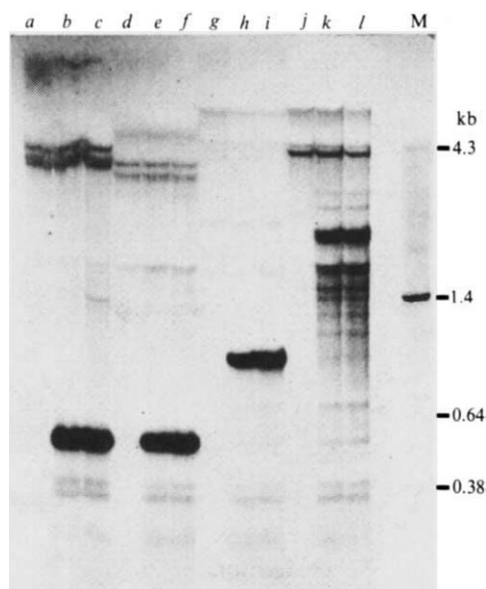


Fig. 3 Mapping and α -amanitin sensitivity of transcripts synthesized *in vitro*. Transcription reactions were carried out as described in Fig. 2 legend, except that 7.5 μ l of extract were used in each reaction mixture. The following DNAs were used as templates: pHB8 cleaved with *Eco*RI (lanes a–c), pHB9.3 cleaved with *Eco*RI (d–f), pHB9.3 cleaved with *Bgl*II (g–i) and pHB9.3 cleaved with *Bam*HI (j–l). Reaction mixtures contained 200 (lanes a, d, g and j), 1 (b, e, h and k) or 0 (c, f, i and l) μ g ml⁻¹ α -amanitin. The 1.4-kb β -globin RNA polymerase II transcript is barely detected at this lysate concentration (lane c); its synthesis, however, is inhibited by a low concentration of α -amanitin, as expected (compare lanes b and c). Accumulation of the 510-nucleotide transcript is inhibited only by the higher concentration, as expected for an RNA polymerase III product (lanes a and b). If the 510-nucleotide RNA does indeed arise from transcription initiation in the β -globin-distal *Alu*I repeat, then the same transcript should be obtained when pHB9.3, which lacks sequences 5' to the β -globin gene (see Fig. 1), is cleaved with *Eco*RI and used as template. Lanes d–f show that this is indeed the case. Further evidence that this RNA initiates at the deduced site was obtained by analysing RNAs transcribed from DNA cleaved with *Hind*III or *Bgl*II. The former results in synthesis of a 630-nucleotide run-off transcript (Fig. 4e–g), while the latter produces a 910-nucleotide transcript (Fig. 3g–i). (The high-molecular weight α -amanitin-resistant species result from end-labelling of DNA.)

Figures 3 and 4 provide further evidence that the 510-nucleotide RNA does in fact arise from transcription initiation by RNA polymerase III in the *Alu*I repeat sequence distal to the β -globin gene. Specifically, the 'run-off' transcription assay¹⁵ was used to map the RNA start site. Transcripts sensitive to high, but not low, levels of α -amanitin and ranging in size from 510 to 910 nucleotides, were detected. These are the sizes expected if transcription begins at the 5' end of the *Alu*I repeat sequence, and continues to the end of the DNA fragment used as templates.

RNA polymerase III, unlike RNA polymerase II, terminates transcription *in vitro* when a stop signal is encountered (for example, see Fig. 2e–h). All *Alu*I-promoted transcripts previously studied terminate at sites within several hundred nucleotides of the repeated element. The largest RNA transcribed from an *Alu*-type repeat previously described is 800 nucleotides¹¹, and all of the approximately 10 other *Alu*-promoted transcripts from the human α - or β -like globin clusters are <600 nucleotides long^{8,9}. It was therefore rather unexpected that transcription actually continued to the end of the *Bgl*II-cleaved DNA (910 nucleotides). To determine the length of the transcribed region, pHB9.3 digested with *Bam*HI was used as template. Surprisingly, the major product of transcription was 2.3 kb, although several minor transcripts (up to 3.2 kb) were detected also (Fig. 3j–l). The transcripts produced from

*Bam*HI-digested DNA arise from specific initiation and termination, as the same RNAs are obtained when closed circular DNA is used as template (Fig. 4a–d).

The function(s) that *Alu*I repetitive sequences serve in cells is unclear, although several possibilities have been discussed (for example, see refs 3 and 9). It is almost certain that at least some of the repeats that are transcribed by RNA polymerase III *in vitro* are also transcriptionally active *in vivo*. For example, specific low-molecular weight transcripts have been shown to be complementary to cloned *Alu*-type repeated DNA sequences isolated from CHO cells¹¹. Also, the observation that the start point of transcription seems to correspond to the first nucleotide of the repeat sequence strongly suggests that the presence of an RNA polymerase III promoter is not coincidental^{16,17}. However, it is unknown if any of the *Alu*I promoters in the β -globin locus are ever used *in vivo*.

The results presented here establish several interesting points with respect to RNA polymerase III and the possible role of *Alu*I repeats. First, RNA polymerase III has long been thought to be the enzyme responsible for synthesis of small RNAs. While most of the products of this polymerase are undoubtedly small (for example, tRNAs, 5S RNA), the results shown above

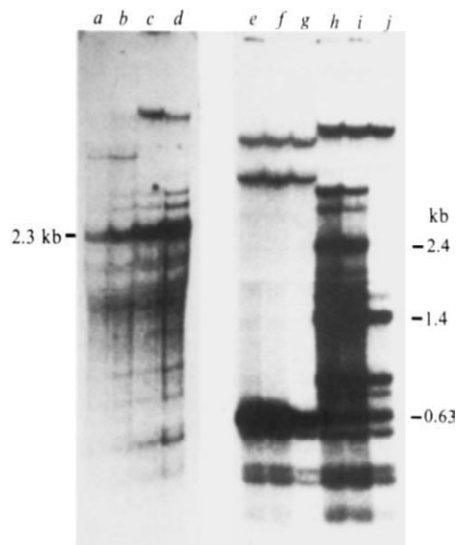


Fig. 4 Comparison of the transcription products obtained from circular and linear DNA. Transcription reactions were as described in Fig. 2 legend except that 7.5 μ l of extract were used. The DNA templates were as follows: pHB9.3 cleaved with *Bam*HI, 60 μ g ml⁻¹ (lane a) or 100 μ g ml⁻¹ (lane b); pHB9.3, Form I, 60 μ g ml⁻¹ (c) or 100 μ g ml⁻¹ (d); pHB8 cleaved with *Hind*III, 100 μ g ml⁻¹ (e–g); and pHB8, Form I, 100 μ g ml⁻¹ (h–j). α -Amanitin (1 μ g ml⁻¹) was included in two reaction mixtures (f and i) and 20 μ g ml⁻¹ α -amanitin was included in two others (lanes g and j). Note that the expected 630-nucleotide run-off transcript is observed with *Hind*III-cleaved DNA (lanes e–g), but the circular DNA gives rise to predominantly larger transcripts (lanes h–j), which arise from termination sites in pBR322. (A stretch of five consecutive T residues is probably responsible for the 2.4-kb RNA, while the 1.4-kb transcript probably results from termination at a stretch of four Ts²³.) These are the only T stretches longer than three in the region of pBR322 that is transcribed, which corresponds, for the most part, to the *tet* gene²⁴. Synthesis of these transcripts is resistant to 1 μ g ml⁻¹ α -amanitin (for example, lanes h and i). However, an interesting response to 20 μ g ml⁻¹ α -amanitin (lane j) was observed: transcription of the smaller RNAs was inhibited only slightly, while synthesis of the larger species was suppressed almost completely. This could reflect a 'target-size' phenomenon in which polymerases that transcribe a greater DNA length have a higher probability of interacting with the drug. Alternatively, α -amanitin may exert its inhibitory effect early during transcription, and those polymerases that continue are more likely to terminate when a potential termination signal is reached, as has been suggested in the case of transcription of 5S genes²³.

establish that this enzyme can synthesize large RNAs, and that templates for such transcription exist in human cells. Second, the possibility that *AluI* repetitive sequences might function by promoting transcription of adjacent genes is strengthened by the observation that a long stretch of DNA can be transcribed. For example, although no precedent exists for transcripts synthesized by RNA polymerase III functioning as mRNA, an RNA 2.3 kb long could encode a protein as large as molecular weight 80,000. Indeed, the presence of *AluI* repeat sequences in cDNA transcripts of polysomal poly(A)⁺ RNA has been reported⁴. Third, since it has been shown that the *AluI* repetitive

sequence studied here is immediately adjacent to a long (~6 kb) middle repetitive DNA sequence (~5,000 copies per genome)^{18,19}, we have established the novel situation in which a short, highly repetitive DNA sequence provides a promoter which results in the *in vitro* transcription of a substantial portion of a long, middle repetitive sequence. It will be of interest to determine the function of this transcription, and any role it may have in influencing the expression of the neighbouring β -globin-like genes.

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- Houck, C. M., Rinehart, F. P. & Schmidt, C. W. *J. molec. Biol.* **132**, 289–306 (1979).
- Rubin, C. M., Houck, C. M., Deininger, P. L., Friedman, T. & Schmid, C. W. *Nature* **284**, 372–375 (1980).
- Jelinek, W. R. *et al. Proc. natn. Acad. Sci. U.S.A.* **77**, 1398–1402 (1980).
- Tashima, M. *et al. Proc. natn. Acad. Sci. U.S.A.* **78**, 1508–1512 (1981).
- Fritsch, E. F., Lawn, R. M. & Maniatis, T. *Cell* **19**, 959–972 (1980).
- Coggins, L. W. *et al. Nucleic Acids Res.* **8**, 3319–3334 (1980).
- Lauer, J., Shen, C. K. J. & Maniatis, T. *Cell* **20**, 119–130 (1980).
- Duncan, C. *et al. Proc. natn. Acad. Sci. U.S.A.* **76**, 5095–5099 (1979).
- Fritsch, E. F., Shen, C. K. J., Lawn, R. M. & Maniatis, T. *Cold Spring Harb. Symp. quant. Biol.* **45**, 761–775 (1980).
- Pan, J., Elder, J. T., Duncan, C. & Weissman, S. M. *Nucleic Acids Res.* **9**, 1151–1170 (1981).
- Haynes, S. R. & Jelinek, W. R. *Proc. natn. Acad. Sci. U.S.A.* **78**, 6130–6134 (1981).
- Proudfoot, N. J., Shatner, M. H. M., Manley, J. L., Gefter, M. L. & Maniatis, T. *Science* **209**, 1329–1336 (1980).
- Manley, J. L., Fire, A., Cano, A., Sharp, P. A. & Gefter, M. L. *Proc. natn. Acad. Sci. U.S.A.* **77**, 3855–3859 (1980).
- Küpper, H., Contreras, R., Khorana, H. G. & Landy, A. in *RNA Polymerase* (eds Losick, R. & Chamberlin, M.) 473–484 (Cold Spring Harbor Laboratory, New York, 1976).
- Weil, P. A., Luse, D. S., Segall, J. & Roeder, R. G. *Cell* **18**, 469–484 (1979).
- Duncan, C. H., Jagadeeswaran, P., Wang, R. R. C. & Weissman, S. M. *Gene* **13**, 185–196 (1981).
- Elder, J. T., Pan, J., Duncan, C. H. & Weissman, S. M. *Nucleic Acids Res.* **9**, 1171–1189 (1981).
- Kaufman, R. *et al. Proc. natn. Acad. Sci. U.S.A.* **77**, 4229–4233 (1980).
- Adams, J. W. *et al. Nucleic Acids Res.* **8**, 6113–6127 (1980).
- Manley, J. L. & Gefter, M. L. in *Gene Amplification and Analysis* Vol. 2 (eds Chirikjian, J. G. & Papas, T. S.) 369–383 (Elsevier-North Holland, New York, 1981).
- Fowlkes, D. M. & Shen, T. *Cell* **22**, 405–413 (1980).
- Hu, S.-L. & Manley, J. L. *Proc. natn. Acad. Sci. U.S.A.* **78**, 820–824 (1981).
- Bogenhagen, D. F. & Brown, D. D. *Cell* **24**, 261–270 (1981).
- Sutcliffe, J. G. *Cold Spring Harb. Symp. quant. Biol.* **43**, 77–90 (1978).

Sequence and structural homologies among type I and type II interferons

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Interferons are proteins with antiviral, antitumour and immunomodulator activities, which are secreted in response to various inducers¹. The interferons have been classified into two categories on the basis of their biological and physical properties. Type I interferons include fibroblast interferon (IFN- β)^{2,3} and the leukocyte family of interferons which is composed of at least 10 subspecies^{4–8}. Each member of the type I interferons contains ~165 amino acids, is acid-stable, and competes for the same receptors⁹; furthermore, identical amino acids occupy invariant positions in 23% of their amino acid sequences⁷. In contrast, type II interferon (IFN- γ) is produced in response to mitogens and antigenic stimuli¹⁰, contains ~146 amino acid residues¹¹, is not acid-stable, and displays no measurable binding to type I interferon receptors⁹. The nucleotide sequence of the cDNA coding for IFN- γ has recently been reported¹¹, and no statistically significant sequence homology was detected between the deduced amino acid sequences of IFN- γ and any of the type I interferons^{11,12}. To determine whether there might be structural similarities between IFN- γ and the type I interferons, we have conducted a predictive analysis of the secondary structure of these proteins^{13,14}. IFN- γ as well as each of the type I interferons contain a segment with a high potential to form an amphiphilic α -helix of approximately the same length and hydrophobic/hydrophilic balance (Fig. 1). As we report here, the identification of these segments generates an alignment of sequences which reveals previously undetected sequence homologies among the type I and II interferons (Fig. 2). Thus, we propose that there is a common evolutionary ancestor for both type I and type II interferons.

Phylogenetic relationships among proteins are often elucidated by demonstrating that the protein sequences may be aligned so that a larger number of identical amino acids occupy respective positions along the polypeptide chain (homologies) than might be expected from a chance alignment of unrelated

proteins. However, when the relationships are distant, deletions (gaps) or additions of residues may occur between homologous regions in the amino acid sequences of the proteins. This hampers the proper alignment of two marginally related sequences because the introduction of gaps increases the number of homologies which would be expected by chance alignment of sequences¹⁵, and also increases by orders of magnitude the number of possible alignments which must be searched for homologies. Clearly, a nonrandom method which would direct the proper alignment of portions of the sequences of related proteins would be extremely useful. When the three-dimensional structures of two proteins have been solved by

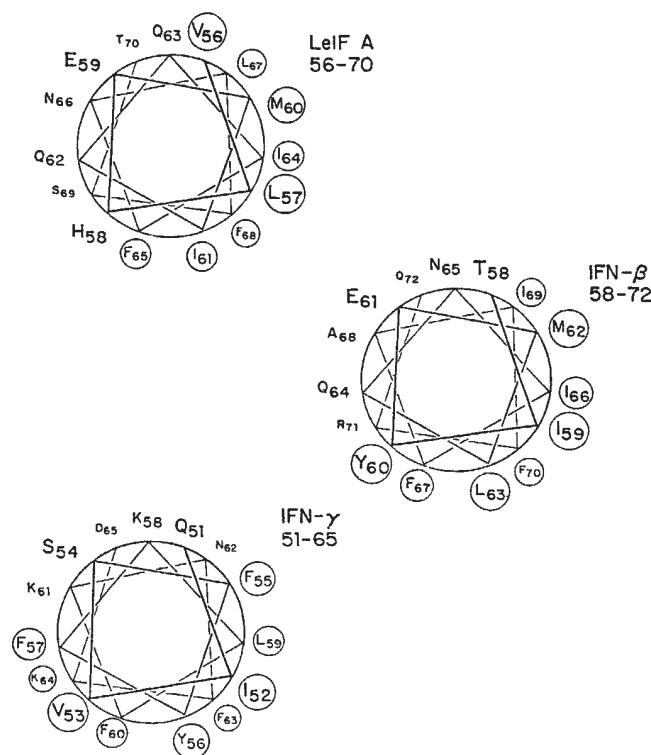


Fig. 1 Axial helical projection¹⁹ of LeIF A⁷ (56–70), IFN- β ³ (58–72) and IFN- γ ¹¹ (51–65).

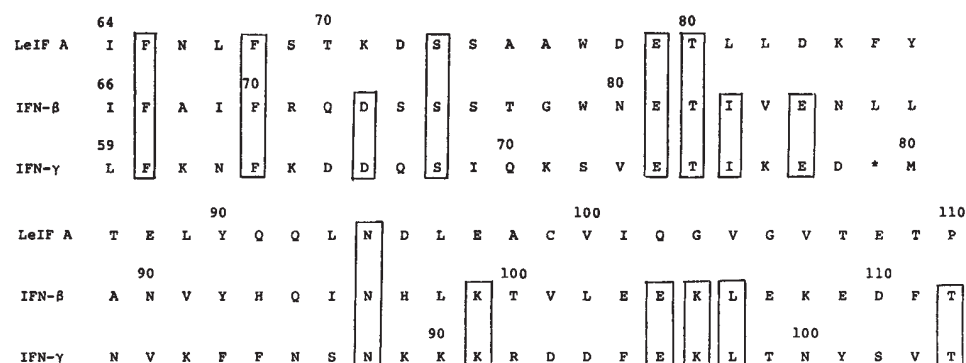


Fig. 2 Amino acid sequences of LeIF A⁷ (63-104), IFN-β³ (65-106) and IFN-γ¹¹ (59-106). Homologous residues are boxed.

X-ray crystallography, relatedness can be demonstrated by aligning the primary structures based on secondary and tertiary structural similarities between the proteins^{16,17}. Unfortunately, the three-dimensional structures of most proteins are not known so we are attempting to make alignments based on predicted secondary-structural similarities. However, most existing methods for secondary structure prediction do not allow precise enough predictions of the positions of initiation or termination of a given secondary structural element¹⁸ to allow unambiguous alignments. Some of these ambiguities might be eliminated by considering the distribution of hydrophobic and hydrophilic residues within a predicted secondary structure. In particular, amphiphilic α -helices have hydrophobic and hydrophilic residues on opposite faces of the α -helix and are known to occur on the surfaces of globular proteins¹⁹ and lipoproteins^{20,21}. Furthermore, the occurrence of amphiphilic α -helices may be predicted by locating the portions of amino acid sequences whose hydrophobic and hydrophilic residues repeat in a pattern such that they are segregated on opposite sides of a helix when the chain adopts a helical conformation^{19,22}. Thus two or more protein sequences might be aligned at sub-sequences which share a hypertensional to form amphiphilic α -helices of similar length and hydrophobic/hydrophilic balance.

LeIF A, IFN-β and IFN-γ have several segments which are predicted to form amphiphilic α -helices (LeIF A was chosen as representative (Fig. 3) of the leukocyte interferons). Of the amphiphilic α -helical segments in these proteins, one in particular is of uniform length and uniform hydrophobic/hydrophilic balance among all three interferons. These segments, which span residues 56-70 in LeIF A, 58-72 in IFN-β and 51-65 in IFN-γ (Fig. 1), also have high potentials to form α -helices as determined by the Chou and Fasman¹⁴ parameters ($\alpha = 1.09, 1.10$ and 1.04 , respectively). When the sequences of the interferons are aligned so that these regions with high amphiphilic α -helical potential coincide, sequence homologies are observed between residues 64-110 (LeIF A), 66-112 (β) and 59-104 (γ). If a space is inserted between positions 79 and

80 of IFN-γ, IFN-γ and IFN-β share 14 invariant residues between residues 59 and 104 (γ numbering), and an additional 4 residues are structurally conserved (Leu for Ile; Lys for Arg; Asp for Asn; Tyr for Phe). Thus, there is 30% sequence homology between β and γ interferon (Fig. 2) in this region and 39% homology if conservative replacements are included; the corresponding values for the homologies between IFN-γ and LeIF A are 13% and 24%, respectively. Similar degrees of homology are observed between IFN-γ and the other leukocyte interferons (Fig. 3).

The significance of our findings was tested by examining the degree of homology to be expected between randomized sequences of IFN-γ and the type I interferons²³. A program was written which performed the following operations: (1) Two random sequences were generated with the frequency of occurrence of amino acids identical to that of the proteins under consideration. (2) A segment of desired length m was selected from the first random sequence. (3) This segment was overlaid on the first m residues of the second (longer) sequence of length n and the number of matches determined. (4) A single gap was inserted into the pair of sequences and the number of homologies noted. (5) Step 4 was repeated for all $2(m-1)$ possible locations of the gap. (6) The segment of length m was moved one residue to the right and steps (3), (4) and (5) repeated. (7) Step 6 was repeated until the end of the longer sequence was reached.

The maximum number of homologies observed for the $2(m-1)(n-m+1)$ possible alignments generated by the above process was noted. Steps (1)-(7) were repeated 1,000 times. The mean and standard deviation of the maximum number of homologies observed were computed. The quantities provide a measure of the degree of homology to be expected for the best alignment (that alignment with the highest number of homologies among all possible alignments) of a segment of m residues on another segment of n residues (with one gap) when the amino acids are randomly distributed. Using this program, we find that the mean and standard deviation for the number of

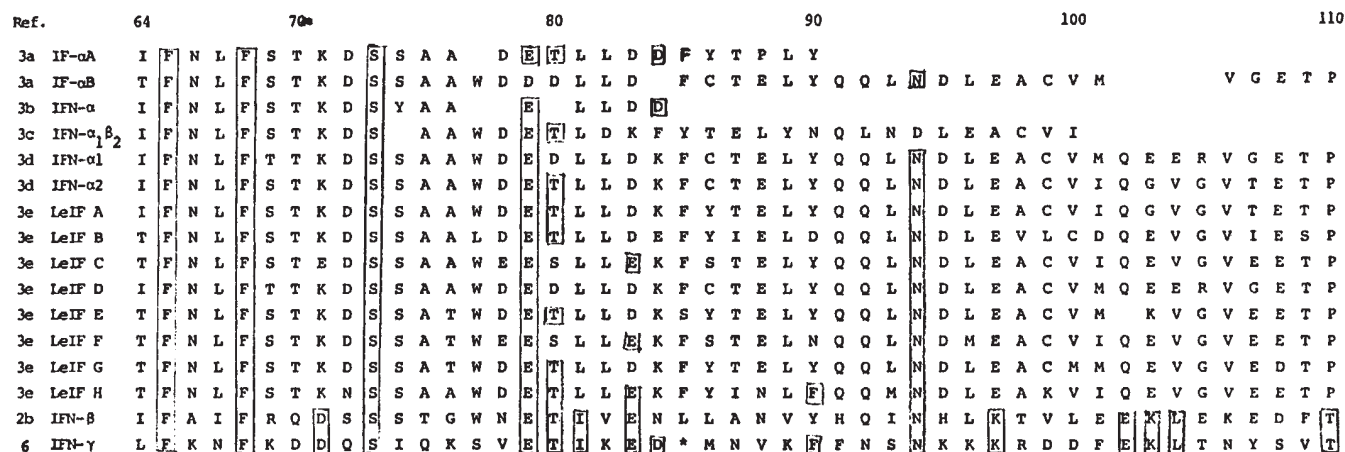


Fig. 3 Partial amino acid sequences of all human interferons thus far reported in the literature. Homologous residues are boxed.

homologies between randomized sequences of IFN- β and a 46-residue segment of IFN- γ is 9.0 ± 1 . Further, the frequency of finding 14 or more homologies is less than 10^{-4} . Notably, although 6 homologies between LeIF A and IFN- γ (Fig. 2) are not statistically significant, these homologous residues are also conserved in IFN- β . The frequency of finding 6 homologies within the 14 homologous positions contained in randomized 46-residue sequences with the amino acid composition of LeIF A and IFN- γ is 4×10^{-3} . (A gap was not included in this calculation because the position of the gap was determined by the prior positioning of the β and γ sequences. If a gap is included the probability is 1.32×10^{-2} .) We conclude that the type I and type II interferons are indeed related and probably arose from a common ancestor.

In contrast to our proposed sequence alignment between IFN- γ and LeIF A, Epstein¹² observed only 14 sequence homologies spread throughout the entire sequences of these two proteins. This is below the value expected for the best random alignment of the entire sequences and insertion of a single gap (16.9 ± 1.7), suggesting that the statistical relatedness of this alignment is far less than the alignment suggested by us.

Our results clearly show the potential for using predicted structural features to align distantly related sequences. This method has been used to demonstrate that a feature common to all interferons thus far sequenced is a segment of 15 residues with a high potential to form an amphiphilic α -helix followed by a region of high sequence homology. The specific amino acid sequence of a large portion of these segments with high amphiphilic potential appears to be of little consequence as long as its amphiphilic character is conserved, suggesting that this region might serve a structural role in stabilizing the surface of these proteins. Zav'yalov and Denesyuk²⁴ and Sternberg and Cohen²⁵ have also suggested that this region is helical and might serve a structural role in the α - and β -interferons.

The degrees of homology between residues 59 and 104 (IFN- γ numbering) among IFN- γ , IFN- β and LeIF A suggest that the IFN- γ gene separated from the IFN- β gene some time after the separation between leukocyte and fibroblast interferon genes. The lower degree of homology between IFN- γ and LeIF A than between IFN- β and LeIF A clearly shows that the mutation rate for IFN- γ has been greater than that for the leukocyte interferons and IFN- β , consistent with type I interferon having separate receptors from the IFN- γ receptor⁹. The observation that the highest concentration of homologies is in a 46-residue portion of IFN- γ and IFN- β suggests that IFN- γ might be a product of the insertion of a short segment of DNA coding for IFN- β into a gene coding for an unrelated protein. IFN- γ gene contains one or more introns¹¹, providing a possible mechanism for this insertion.

We thank E. Knight for helpful discussions.

Since submission of this paper the complete sequence of the human immune interferon (IFN- γ) gene including the exon and intron structure has been published²⁶. A single exon of 61 amino acids in length contains the entire segment predicted by us here to form an amphiphilic α -helix as well as 44 of 46 amino acids we also show to be homologous. Furthermore, only 9 of the 61 amino acids in this exon do not appear in the structurally and functionally homologous unit proposed by us. This observation further supports previous suggestions^{27,28} that exons code for functionally competent domains of proteins.

13. Edelstein, D., Kézdy, F. J., Scanu, A. M. & Shen, B. W. *J. Lipid Res.* **20**, 143–153 (1979).
14. Chou, P. Y. & Fasman, G. D. *Adv. Enzym.* **47**, 45–148 (1978).
15. Haber, J. E. & Koshland, D. E. Jr *J. molec. Biol.* **50**, 617–639 (1970).
16. James, M. N. G., Delbaere, L. T. J. & Brayer, G. D. *Can. J. Biochem.* **56**, 396–402 (1978).
17. Keim, P., Heinrichson, R. L. & Fitch, W. M. *J. molec. Biol.* **151**, 179–197 (1981).
18. Matthews, B. W. *Biochim. biophys. Acta* **405**, 442–451 (1975).
19. Shiffer, M. & Edmundson, A. B. *Biophys. J.* **7**, 121–135 (1967).
20. Segrest, J. P., Jackson, R. L., Morrisett, J. D. & Gotto, A. M. Jr *FEBS Lett.* **38**, 247–253 (1974).
21. Fukushima, D. et al. *J. Am. chem. Soc.* **101**, 3703–3704 (1979).
22. Lim, V. I. *J. molec. Biol.* **88**, 857–872 (1974).
23. Doolittle, R. F. *Science* **214**, 149–214 (1981).
24. Zav'yalov, V. P. & Danesnyuk, A. I. *Immun. Lett.* **4**, 7–14 (1982).
25. Sternberg, J. E. & Cohen, F. E. *Int. J. biol. Macromolec.* **4**, 137–144 (1982).
26. Gray, P. W. & Goeddel, D. V. *Nature* **298**, 859–863 (1982).
27. Gilbert, W. *Nature* **271**, 501 (1978).
28. Craik, C. S., Buchman, S. R. & Beychok, S. *Proc. natn. Acad. Sci. U.S.A.* **77**, 1384–1388 (1980).

Nucleotide sequence of $\gamma\delta$ resolvase gene and demonstration that its gene product acts as a repressor of transcription

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The related transposons $\gamma\delta$ and Tn3 each encode an efficient site-specific recombination system essential for transposition^{1,2}. The recombination occurs at a particular site, *res*, located within the intercistronic region between the two divergently transcribed genes that are required for transposition². The product of one of these genes, resolvase, catalyses the recombination at *res*. Recently, *in vitro* site-specific recombination has been demonstrated using purified resolvase and a model substrate containing two *res* sites³. Genetic analysis of mutants in the $\gamma\delta$ /Tn3 *tnpR* gene, which encodes the resolvase protein, suggested that resolvase regulates the frequency of transposition in addition to catalysing recombination^{4–6}. Analysis of element-encoded proteins in minicells indicated a direct effect of resolvase on the levels of the element-encoded transposase, as well as on its own expression⁷. Here we report the sequence of the gene encoding $\gamma\delta$ resolvase and compare the predicted amino acid sequence with that of the functionally interchangeable Tn3 protein⁶. In addition, we have determined the sites at which transcription of the transposase and resolvase genes initiates *in vitro* and have demonstrated that purified resolvase protein specifically represses transcription of both transposition related genes in this *in vitro* transcription system.

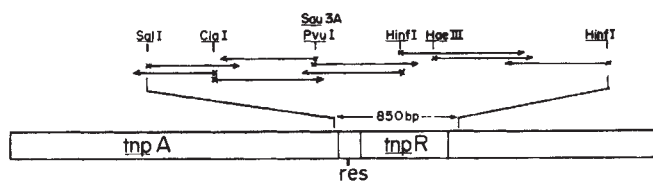


Fig. 1 Genetic map of $\gamma\delta$ and the region sequenced. The element is drawn with the γ end¹⁷ towards the right. Transcription of *tnpA* and *tnpR* is divergent from the central intercistronic region. The right arm appears to encode a 79,000 molecular weight polypeptide of unknown function (unpublished observation). The locations and directions of sequence determinations are indicated by arrows with the 5' labelled end indicated by X. The DNA was labelled and sequenced as described by Maxam and Gilbert¹⁸. The $\gamma\delta$ *tnpR* sequences were determined using fragments from the plasmid pOX14, a $\gamma\delta$ transposition into pBR322¹⁷.

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1. Stewart, W. E. II *The Interferon System* (Springer, New York, 1979).
2. Knight, E. Jr. et al. *Science* **206**, 525–526 (1980).
3. Taniguchi, T., Ohno, S., Fuji-Kuriyama, Y. & Muramatsu, M. *Gene* **10**, 11–15 (1980).
4. Allen, G. & Fantes, K. H. *Nature* **287**, 408–411 (1980).
5. Zoon, K. C. in *The Biology of the Interferon System* (eds deMaeyer, E., Galasso, G. & Schellekens, H.) 47–55 (Elsevier, Amsterdam, 1981).
6. Levy, W. P. et al. *Proc. natn. Acad. Sci. U.S.A.* **78**, 6186–6190 (1981).
7. Goeddel, D. V. et al. *Nature* **290**, 20–26 (1981).
8. Streuli, M., Nagata, S. & Weissman, C. *Science* **209**, 1343–1347 (1980).
9. Branca, A. A. & Baglioni, C. *Nature* **294**, 768–770 (1981).
10. Morris, A. G., Lin, Y.-L. & Askonas, B. A. *Nature* **295**, 150–152 (1982).
11. Gray, P. W. et al. *Nature* **295**, 503–508 (1982).
12. Epstein, L. E. *Nature* **295**, 453–454 (1982).

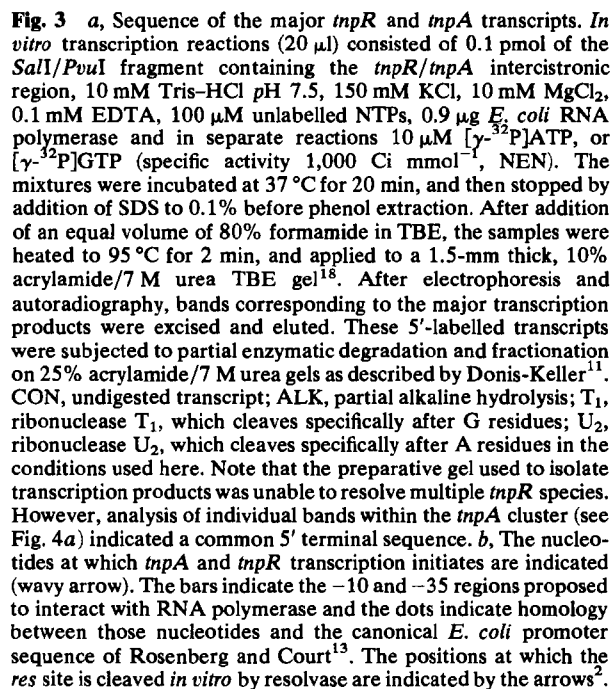
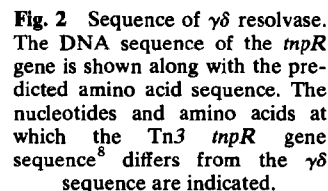


Fig. 4 *a*, Specific repression by $\gamma\delta$ resolvase. Transcription was performed as in Fig. 3 except that [α - 32 P]GTP (specific activity 50 Ci mmol $^{-1}$; NEN) was used. As an internal control, the efficiency of transcription 0.1 pM of a 165 bp fragment containing the spot 42 promoter and terminator (C. N. Joyce, personal communication) was also monitored in each reaction. Lanes 1–5 contain both templates, lane 6 only the *res* template and lane 7 only the spot 42 template. Resolvase was added and the reactions incubated for 5 min before the addition of RNA polymerase. Lane 1, 0.1 pmol resolvase; lane 2, 0.3 pmol resolvase; lane 3, 0.6 pmol resolvase; lane 4, 2.0 pmol resolvase; and lanes 5–7, no added resolvase. The identity of each indicated transcript was confirmed by two-dimensional T₁-ribonuclease fingerprinting and by secondary analysis¹⁹. The multiple bands result from heterogeneity at the 3' ends of the run-off transcripts. The band labelled (*tnpR*) is a prematurely terminated *tnpR* transcript. *b*, Densitometric analysis of transcriptional repression by resolvase. Lanes 1–5 were scanned and the intensities of each peak determined relative to that in the absence of resolvase (lane 5). The results were then plotted versus the molar ratio of resolvase monomers to *res* template. Spot 42 transcription is indicated by $\circ$, *tnpR* by $\square$ and *tnpA* by $\triangle$. Decreases in intensity were identical for each band within the *tnpA* cluster.

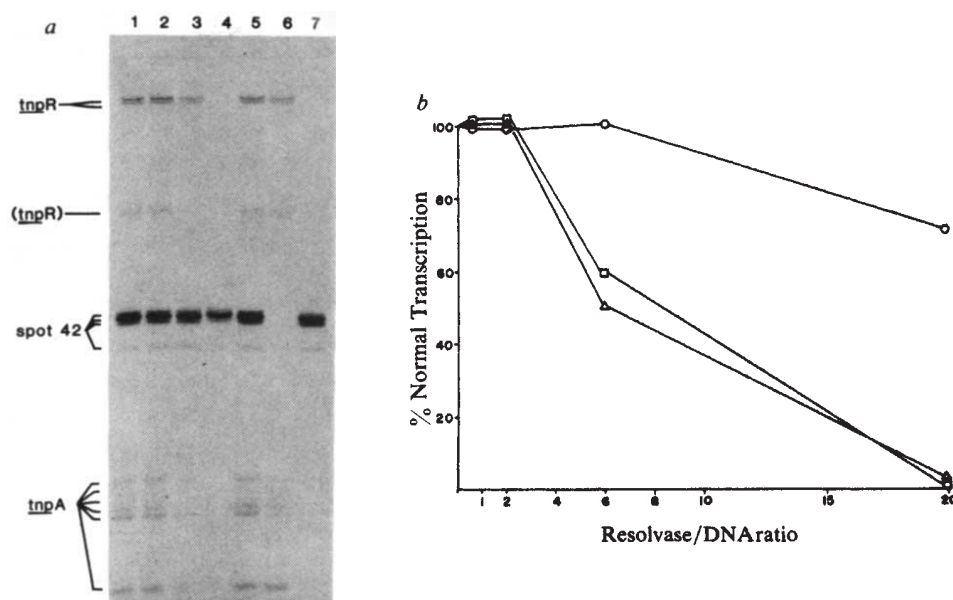


Figure 1 shows a map of $\gamma\delta$ and of the region sequenced. The DNA sequence has revealed a single open reading frame which is capable of encoding a slightly basic 183-residue polypeptide, corresponding to the $\gamma\delta$ resolvase protein (Fig. 2). The amino acid sequence of $\gamma\delta$ resolvase shows 80% homology with that of the analogous Tn3 protein⁸, the homology being particularly extensive for the N-terminal two-thirds of the sequence. In view of the interchangeability of the $\gamma\delta$ and Tn3 proteins in both recombination and repressor roles², the high degree of homology in the N-terminal region may indicate a conservation of the DNA binding site. Interestingly, the protein which catalyses recombination in the *hin* site-specific recombination system of *Salmonella typhimurium* (which is involved in the expression of flagellar proteins)⁹ is homologous to $\gamma\delta$ resolvase, and exhibits a similar pattern of sequence conservation. Resolvase and the *hin* protein are not interchangeable for recombination functions (unpublished observation).

Considerable genetic evidence has suggested that $\gamma\delta$ /Tn3 resolvase regulates expression of element-encoded proteins (resolvase and transposase) at the transcriptional level¹⁰. We have determined the sites at which transcription of these two genes initiates *in vitro*. A purified 303 bp *SalI*/*PvuI* fragment containing all of the intercistronic region between *tnpA* and *tnpR* was used in transcription experiments with *Escherichia coli* RNA polymerase and γ - 32 P labelled purine triphosphates. The resulting transcripts were sequenced by the method of Donis-Keller¹¹ (see Fig. 3a). Determination of the two start sites allows identification of the regions of the DNA sequence suggested to be involved in RNA polymerase interactions¹² (Fig. 3b); homologies between these regions and the canonical *E. coli* promoter sequence¹³ are indicated by dots. The -10 region of the two promoters overlap one another, and contain the site at which resolvase cleaves the *res* site to initiate strand exchange¹⁴ (indicated by the arrows in Fig. 3b). This cleavage is observed at a significant frequency only in special conditions and thus is unlikely to have a role in regulation.

The divergent transcription of regulatory proteins and of the genes they control has been described previously. However, in the *araC/araBAD* operons of *E. coli*¹⁵ as well as in the rightward region of bacteriophage λ (ref. 16) the nucleotides at which divergent transcription initiates are separated by more than 50 base pairs (bp). In the *res* system, RNA polymerase

competition for binding at the two promoters may represent an additional regulatory complexity.

Genetic evidence has suggested a role for resolvase in regulation of the *tnpR* and *tnpA* genes. The data in Fig. 4 demonstrate that transcription from the *SalI*/*PvuI* fragment used in the previous experiment can be specifically repressed by the addition of resolvase. As an internal control, we monitored the efficiency of transcription of a promoter-containing fragment not expected to be regulated by resolvase (the spot 42 transcript). The resolvase concentration at which a significant reduction of transcription of the *SalI*/*PvuI* fragment occurred (~6 protein monomers per template) corresponds closely to that required for resolution (~6–8 monomers per resolution site)³. *In vitro* recombination studies have identified the two sites at which resolvase mediates strand cleavage to initiate recombination¹⁴. Their location within the Pribnow boxes for *tnpA* and *tnpR* transcription strongly suggests that resolvase regulates expression of transposition functions and resolves intermediates in the transposition pathway by acting at a single site or group of sites located within the intercistronic region between the divergently transcribed transposase and resolvase genes.

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1. Arthur, A. & Sherratt, D. J. *Molec. gen. Genet.* **175**, 267–274 (1979).
2. Reed, R. *Proc. natn. Acad. Sci. U.S.A.* **78**, 3428–3432 (1981).
3. Reed, R. R. *Cell* **25**, 713–719 (1981).
4. Chou, J., Casadaban, M. J., Lemaux, P. G. & Cohen, S. N. *Proc. natn. Acad. Sci. U.S.A.* **76**, 4020–4024 (1979).
5. Chou, J., Lemaux, P. G., Casadaban, M. J. & Cohen, S. N. *Nature* **282**, 801–806 (1979).
6. Kitts, P. A., Symington, L., Burke, M., Reed, R. & Sherratt, D. *Proc. natn. Acad. Sci. U.S.A.* **79**, 46–50 (1982).
7. Gill, R., Heffron, F. & Falkow, S. *Nature* **282**, 797–801 (1979).
8. Heffron, F., McCarthy, B. J., Ohtsubo, H. & Ohtsubo, E. *Cell* **18**, 1153–1163 (1979).
9. Zieg, J. & Simon, M. *Proc. natn. Acad. Sci. U.S.A.* **77**, 4196–4200 (1980).
10. Kitts, P. A., Lamond, A. & Sherratt, D. J. *Nature* **295**, 626–628.
11. Donis-Keller, H., Maxam, A. & Gilbert, W. *Nucleic Acids Res.* **4**, 2527–2538 (1977).
12. Siebenlist, U., Simpson, R. B. & Gilbert, W. *Cell* **20**, 269–281 (1980).
13. Rosenberg, M. & Court, D., *A. Rev. Genet.* **13** (1979).
14. Reed, R. & Grindley, N. D. F. *Cell* **25**, 721–728 (1981).
15. Lee, N. L., Gielow, W. O. & Wallace, R. G. *Proc. natn. Acad. Sci. U.S.A.* **78**, 752–758 (1982).
16. Ptashne, M. *et al. Cell* **19**, 1–12 (1980).
17. Guyer, M. J. *molec. Biol.* **126**, 346–365 (1978).
18. Maxam, A. & Gilbert, W. *Meth. Enzym.* **65**, 499–560 (1980).
19. Barrell, B. G. in *Proceedings in Nucleic Acids Vol. 2* (eds Cantoni, G. L. & Davis, D. R.) 751–779 (Harper & Row, New York, 1971).

MATTERS ARISING

Porphyropsin in retina of four-eyed fish, *Anableps anableps*

A RECENT investigation¹ reports the same visual pigments in the dorsal and ventral regions of *Anableps* retina. I present here some previously unpublished studies that demonstrate the existence of porphyropsin as well as rhodopsin in this species. *Anableps* is therefore similar to two related poeciliids, *Belonesox belizanus* and *Mollienesia latipinna*². The *Anableps* used in the present work were examined only 3 days after collection from their native waters in Guiana, whereas the fish used by Avery and Bowmaker¹ originated from a fish supplier in London. Mixed rhodopsin-porphyrpsin retinas are highly variable in composition; this variability probably affects the cone system also³ and often depends on environmental factors⁴. For example, it has been shown that *Belonesox* may lose much of its porphyropsin if kept in unnaturally bright surroundings⁵ and that its rhodopsin-porphyrpsin balance shifts markedly with season and local habitat⁶. Other species are known to behave similarly⁴. Therefore, Avery and Bowmaker's results do not exclude the possibility that in its natural habitat *Anableps* may have appreciable proportions of porphyropsin that could be segregated in its dorsal retina.

Specimens of *Anableps* were flown directly from Guiana to Miami. A total

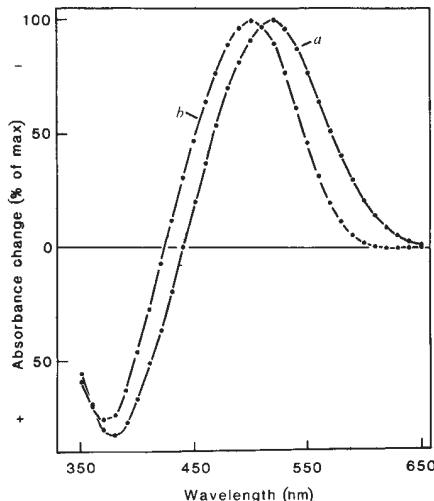


Fig. 1 Analysis of *Anableps* visual pigments. Curve *a* represents the difference spectrum obtained by bleaching a digitonin extract with light of wavelength 660 nm in the equipment described by Bridges²: the maximum absorbance loss was 0.077 at 519 nm. After several subsequent exposures to red light (660 and 620 nm), the visual pigment was completely bleached by exposure to yellow light (>440 nm). Curve *b* represents the difference spectrum for this final irradiation: the maximum absorbance loss was 0.0944 at 500 nm (see text for details).

of 3 days elapsed between the time of netting and delivery (August 1963) to my laboratory in the Bascom Palmer Eye Institute, University of Miami, Florida. After overnight dark-adaptation at 80°F, 11 fish ranging in standard length from 7 to 10 cm were killed by decapitation. The retinas were then dissected out and the photoreceptor outer segments prepared by sucrose flotation². Further procedures used have been described previously². The visual pigment was extracted in three successive 0.5-ml volumes of 2% digitonin (Merck), and each extract was buffered to pH 8.8 with sodium borate.

The data presented here are for the first extract from the above procedure, which had a peak absorbance of 0.57 at 507 nm. The extract had more than one visual pigment—this was demonstrated by exposing it to a series of irradiations at wavelengths ranging from 660 to 620 nm, then finally to non-isomerizing yellow light of wavelengths >440 nm. Figure 1 curve (*a*) shows the difference spectrum for an initial exposure to 660 nm light. The amount bleached represents 14% of the total visual pigment, and the difference spectrum has a $\lambda_{\max}$ at 519 nm, close to that of porphyropsin. The difference spectrum for the final exposure to yellow light (representing 16% of the total pigment) had a $\lambda_{\max}$ at 500 nm, typical of rhodopsin. As Fig. 1 shows, the $\lambda_{\max}$ of the bleaching product also shifted to shorter wavelengths. A single fish from this group gave similar results: the digitonin extract had a peak absorbance of 0.12 at 506 nm. As the cone pigments of fishes do not survive digitonin extraction, it seems that the *Anableps* used here contained a mixture of rhodopsin and porphyropsin in their retinas.

The $\lambda_{\max}$ of *Anableps* rhodopsin was calculated⁴ to be identical to that reported for *Belonesox*² that is, 498 nm, and the proportion of porphyropsin was 25–30%. The presence of porphyropsin may have accounted for the elevated $\lambda_{\max}$ of 506 nm previously assigned to the rhodopsin of this species⁷. The apparent agreement with Avery and Bowmaker's mean rod $\lambda_{\max}$ may be fortuitous, because *in situ* values are often displaced to longer wavelengths^{8,9}.

Thus, *Anableps* appears to be similar to several of its close relatives in having a mixture of rhodopsin and porphyropsin. Extensive studies of other species have established that such mixtures may vary widely in composition, and that ambient illumination is an important controlling factor⁴. Therefore, further studies of *Anableps* in its natural habitat or in a variety of controlled lighting conditions are needed before a final conclusion on its dorso-ventral distribution of visual pigments can be reached.

This work was supported in part by

grants from the NSF and the NIH (National Eye Institute).

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1. Avery, J. A. & Bowmaker, J. K. *Nature* **298**, 62–63 (1982).
2. Bridges, C. D. B. *Vision Res.* **5**, 223–238 (1965); *Nature* **203**, 303–304 (1964).
3. Tsien, A. T. C., Liebman, P. A., Beatty, D. D. & Drzymala, R. *Vision Res.* **21**, 943–946 (1981).
4. Bridges, C. D. B. in *Handbook of Sensory Physiology Vol. 7/1* (ed. Dartnall, H. J. A.) 417–480 (Springer, Berlin, 1972).
5. Bridges, C. D. B. *Nature* **206**, 1161–1162 (1965).
6. Bridges, C. D. B. *Nature* **203**, 191–192 (1964).
7. Schwanzara, S. A. *Vision Res.* **7**, 121–148 (1967).
8. Knowles, A. & Dartnall, H. J. A. in *The Eye Vol. 2B* (ed. Davson, H.) 347–423 (Academic, New York, 1977).
9. Bowmaker, J. K. *Vision Res.* **13**, 783–792 (1973).

AVERY AND BOWMAKER REPLY— Bridges presents data from partial bleaching experiments to show that the rod pigment of *Anableps* ($\lambda_{\max}$ = 506–507 nm) is actually a mixture of rhodopsin ($\lambda_{\max}$ = 498 nm) and a porphyropsin ($\lambda_{\max}$ = 519 nm), and is not a pure rhodopsin as reported by Schwanzara¹. In our recent investigation of the dorso-ventral distribution of visual pigments in *Anableps*², we suggested from the shape of the absorbance spectra of the rods, obtained by microspectrophotometry, that the pigments were probably rhodopsins, but we are not surprised to find that they are rhodopsin-porphyrpsin mixtures.

Bridges suggests that we found no difference between the visual pigments of the dorsal and ventral parts of the retina possibly due to a change in the rhodopsin-porphyrpsin ratio caused by keeping the fish in artificial light. Our value of the mean $\lambda_{\max}$ of the rods is, however, within 1 nm of the value he and Schwanzara¹ obtained for extracts of the rod pigment, inferring that the rhodopsin-porphyrpsin ratio in the rods we measured in both regions of the retina was the same as that of their extracts. If *Anableps* does have higher proportions of porphyropsin in the dorsal retina in its natural habitat, we would have expected the $\lambda_{\max}$ of Bridges' extract to have been longer than our rod mean $\lambda_{\max}$. Unfortunately, extracts normally contain only rod pigments and give no information about the distribution of pigments across the retina or the nature of the cone pigments. Thus, further microspectrophotometric studies are required.

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2. Avery, J. A. & Bowmaker, J. K. *Nature* **298**, 62–63 (1982).

BOOK REVIEWS

Multiple realities?

Marie Jahoda

IN THE last quarter of the nineteenth century psychologists, impressed by the advances in the hard sciences, emulated their big brother and issued a unilateral declaration of independence from philosophy. Both, emulation and independence, were powerful motivators; they appeared to work reasonably well in mainstream psychology, even though doubts about the wisdom of either were raised from the beginning.

For a good part of the twentieth century emulation was based on the mechanistic physics of earlier periods. But physics had undergone revolutionary changes. Its new concepts — particularly Heisenberg's principle of uncertainty and the probabilistic nature of knowledge in many areas — gradually permeated the intellectual culture of psychologists, many of whom were all too aware of uncertainties in their methods, concepts and findings. References to Heisenberg increased in the psychological literature, used defensively however, rather than as a stepping stone to further advances, as was the case in physics.

The book under review, written by a research psychologist and a physicist, repeals the declaration of independence from philosophy in both disciplines and searches for a redefinition of their communalities and differences. It is organized in three parts, entitled "The Meanings of Reality", "The Search for Scientific Truth" and "Domains of the Social Sciences". Philosophers of science have dealt with such issues often as outsiders to the practice of science and have, as a rule, concentrated on the hard sciences; the reversed intrusion by practitioners of science into epistemology is largely limited to a specific field. The increase in books, of which this is one, that transcend both these limitations is a welcome sign of efforts to put the human sciences on a more solid base, even those aspects where matters count that cannot be counted; at least not yet.

In crude outline (omitting many interesting asides such as Heisenberg's appreciative comments on Goethe's theory of colours) the rather involved argument of the authors runs thus: the materialistic concept of reality has been shown to be limited by "the works of Heisenberg, Schrödinger, Einstein, Born, Freud, and Jung" (p.1) and needs reformulation. While common sense still regards as real only the external world that can be seen or touched, modern physics constructs as real

Einstein's Space and Van Gogh's Sky: Physical Reality and Beyond. By Lawrence LeShan and Henry Margenau. Pp.268. ISBN 0-02-570460-5. (Macmillan, New York: 1982.) \$14.95. To be published in the United Kingdom in January 1983 by Harvester.

events in the macro- and in the microcosm that are beyond the sensory competence of the human organism and can be understood only mathematically or through inference from observable consequences. Newton's formulation of reality was not wrong but insufficient in view of later constructions that left room for his but transcended it. In particle physics and astrophysics visualization is no longer possible, but every physicist regards the regularities established in these areas as real. New constructs of reality have been invented and their correspondence to observation has been discovered.

Physics was for a time erroneously believed by some human scientists to have solved their problems. This is wrong. The aspect to be taken on board from modern physics is the idea that it is legitimate and, for certain areas, necessary to use alternate interpretations of reality, particularly when psychologists go beyond overt behaviour to study the domains of inner life. Here it is unavoidable to assume that purpose and intention are real, even though they can only be inferred (much like the electron), not seen or felt by an observer. This is a construction of psychic reality with which many psychologists have been familiar since the beginning of the century in a systematic way, but even St Augustine knew that psychological time had to be construed differently from measurable time, and accepted both as real.

Just as physics distinguishes between various domains (mechanics, quantum physics and so on) that are based on different constructions of reality, so the human sciences must identify their domains. Those suggested here in addition to molar behaviour are: art, parapsychology, ethics and consciousness. A curious assembly.

Ignoring this idiosyncratic choice of domains, what is one to make of all this? As a non-physicist I learn from this and other expositions of modern physics for the layman one important lesson: I have to take it on credit. As a non-philosopher I can only marvel at the amount of mental energy that has been expended over the

centuries on pondering what reality really is: a dream, a unitary or multiple cosmos, a deliberate creation or an evolution by chance and necessity? These eternal ontological questions are metaphysics, not science. As a psychologist, I agree with the authors of this volume, that, whatever one's metaphysical stance "... we can never determine the 'true' shape and order of reality. We shall have to give up this dream" (p.24). All we can do *qua* scientists is to invent different ways of thinking about reality (including consciousness as much as the physical world) and check the extent to which concepts correspond to observation.

Two pages later, on p.26, the various constructs have changed into various essences, when the phrase is "... multiple, equally valid realities ...", not constructs of reality. The very chapter title "Alternate Realities" implies the same deviation from p.24, and throughout the book there are other examples of confounding what there is with how we think about it. Surely we must assume that particles performed their indeterminate dance even before modern physics began to look at them as real?

It may seem pedantic to criticize the authors for what might be regarded only as elliptic phrasing, given that the basic position of an unending asymptotic approach to reality was stated clearly early on. But this is not the only case of loose talk about concepts crucial to the entire argument. Another is the concept of purpose, an indispensable concept in much of psychology where it is regarded as a state of consciousness in the here and now. On p.132, however, it is equated with "determination by the future"; on p.157 "future states influence present occurrences". This is magic. Once again, St Augustine and modern psychology know better. It is the present anticipation, not a future event which may or may not occur, that influences experience and action.

There are also some minor flaws: R.F. Bales is consistently misspelled; the index is not very helpful; some of the down-to-earth examples are not convincing; and to usurp art and ethics for psychology seems to me misplaced imperialism. Altogether, an interesting intention has been disappointingly executed. □

Marie Jahoda is Professor Emeritus of Social Psychology and Consultant to the Science Policy Research Unit at the University of Sussex.

What's what in organic chemistry

E.R.H. Jones

Heilbron's Dictionary of Organic Compounds, 5th Edn. Executive editor J. Buckingham. Seven volumes, pp.7,848. ISBN 0-412-17000-0. (Chapman & Hall/Methuen: 1982.) £975, \$1,950.

THE number of compounds described in the literature provides a more precise measure of the growth of chemistry than publications or other indicators. Organic chemistry, thanks to the tetravalency of carbon and the stability of its incestuous bonds, is responsible for most of these compounds; even the recent plethora of inorganic compounds is attributable to organic coordinating components. The number is now nearly five million and, resulting from advances in techniques, many recent additions have large and complicated structures. Remarkably, however, this situation is being coped with.

Some 50 years ago Professor Ian Heilbron and H.M. Bunbury recognized the need of chemists for something akin to the *Concise Oxford*, and the first edition of the *Dictionary of Organic Compounds* (1934), a most successful venture, provided a readily accessible source of information. "Heilbron" is the first point of enquiry for many, and this fifth edition (the fourth appeared in 1965) is timely indeed. The need for facile reference to the literature has never been greater.

In the new edition the original policy of encompassing all of interest and importance is more specifically defined; the intention has been to include fundamental compounds of simple structure, the majority of important natural products, compounds of commercial value, and laboratory chemicals and those with special chemical, physical or biological properties. Selection of the 50,000 entries, by deletion as well as addition, which overall results in the mention of about 1 in 30 of known compounds, has been satisfactorily achieved with the assistance of a hundred chemists, including some 20 special editors, all experts.

With this highly organized team and using modern methods of data handling, the selection and compilation of information and production of the books were completed within five years so that the primary literature has been covered to the end of 1980. Although the new edition involves "the most radical revision and modernisation", the traditional format, the generous provision of structural formulae and indeed all the familiar features have been retained. And there are significant changes. Citing only one author's name is a sensible economy and specifying the content of references (e.g., isol, synth, struct, cmr and so on) is a valuable innovation, as is the extensive inclusion of toxicity and hazard indications and references.

Organic chemists think in terms of structural formulae and need assistance to translate these into dictionary entries. Help is abundantly available from the molecular formulae and name indexes. Alternative and recommended names are given, indication of their sources is useful and Chemical Abstracts Registry Numbers provide a nomenclature-independent link to, and from, those printed here and to the other services which are increasingly using Registry Numbers. A heteroatom index, in 62 sections, points to the entries containing elements other than C, H, O, N and halogens. Thus we find amongst the 1,500

organo-metallic compounds 19 of copper and 25 of manganese. Accessibility to the alphabetical entries from the indexes is enormously facilitated by serial numbers for each entry, and will be most helpful with the annual supplements (the first promised for 1983).

By comparison with the cost of periodicals and other books, the seven volumes and 7,800 pages represent excellent value for scarce library money. The *Dictionary* is an essential adjunct wherever organic compounds are used, nowhere more so than in educational establishments where students need training to cope with the population explosion in organic chemistry. □

Sir Ewart Jones is Emeritus Professor of Chemistry at the University of Oxford.

The bones of palaeopathology

D.R. Brothwell

Identification of Pathological Conditions in Human Skeletal Remains. By Donald J. Ortner and Walter G.J. Putschar. Pp.479. (Smithsonian Institution Press/US Government Printing Office: 1982.) Pbk \$12.

TWENTY-five years ago, as a young graduate of University College London, I was puzzled and impressed by the near total eclipse of interest in sub-fossil skeletal remains, and especially the evidence of palaeopathology which could be found in them. A quarter of a century earlier, in Egypt and eventually at the same college, Sir Grafton Elliot Smith and colleagues had produced distinguished studies on thousands of skeletons and mummies, paying close attention to the evidence of disease. Yet in my time, bones were a matter of considering the many scraps of fossil hominids or were simply used in the general teaching of comparative vertebrate anatomy.

The balance of interests in the biology of man is ever changing, however, with the result that human disease ecology and even such highly specialized topics as the problems of identifying disease in excavated skeletal remains is receiving renewed attention. Evidence of this, if evidence were needed, is this handsome large-format book by two of the senior names in palaeopathology.

Considering the vast literature on medical diagnosis, one might imagine that there were sufficient references to the differential diagnosis of skeletal disease. What had Elliot Smith, Ruffer, Wood Jones and all the other golden oldies used? The answer is that they made do with rather limited texts and similarly limited dry bone specimens in medical museums, and with a range of clinical experience of bone pathology in those pre-antibiotic times. But

museum displays are changing (if not shrinking) and dry bones are no longer priority exhibits. It was thus becoming critical that some detailed stock-taking was undertaken, preferably with an eventual published text and "atlas" of good illustrations in mind.

These changes then, and the growing literature on bone pathology, demanded an up-to-date review of the subject, preferably with a large range of well-selected, high-quality illustrations. Although a number of introductory texts and two symposium proceedings appeared in the 1960s, the definitive reference work of the kind provided by Ortner and Putschar remained a much needed project. This is a well-illustrated, carefully researched collaborative effort providing a comprehensive review of pathological conditions that affect the human skeleton. Having seen the authors in action, scanning the Royal College of Surgeons collection in London, I can vouch for the care with which they have not only surveyed past literature, but personally examined a very wide range of specimens of known diagnosis. There are thus authoritative details of a wide range of diseases affecting the skeleton, from congenital abnormality, trauma and arthropathy, to endocrine and metabolic disturbances, circulatory disorders and the numerous divisions of infectious disease (including less well known bone pathology, the result of mycotic infections, brucellosis, sarcoidosis and echinococcosis).

There is no doubt that this volume is an essential reference work for all those who handle ancient human skeletal material. It should also hold interest for medical historians as well as radiologists, pathologists and orthopaedic specialists. □

Don Brothwell is Reader in Zooarchaeology at the Institute of Archaeology, London.

Plants and biochemical defence

John Friend

Phytoalexins. Edited by John A. Bailey and John W. Mansfield. Pp.334. ISBN 0-216-91162-1. (Blackie/Halsted: 1982.) £28, \$79.95.

THE existence of phytoalexins as anti-fungal compounds actively accumulated by plants was first proposed in 1940 to explain the response of potato tuber tissue to inoculation with avirulent and virulent races of *Phytophthora infestans*. Although the major sesquiterpenoid phytoalexin from potato was not isolated until 1968, in the intervening period phenolic and terpenoid phytoalexins were identified in orchids, sweet potato, carrots, peas and French beans. Many more plants have since been investigated for their ability to produce phytoalexins in response to environmental stress, as well as to challenge not only by fungi but also by bacteria and viruses and by salts of heavy metals.

The aim of the editors of *Phytoalexins* was to review in detail the compass of current research into these compounds; with the aid of a battery of international authorities on the various topics, this is what they have done and excellently so. Broadly the book falls into two parts, the first of which deals mainly with the chemical study of phytoalexins.

Since the majority of such compounds have been isolated from the Leguminosae and the Solanaceae a separate chapter is devoted to each of these families (by J.L. Ingham and J. Kuć, respectively), whereas the phytoalexins from all the other families are covered in a further contribution by D.T. Coxon. Ingham's chapter is particularly valuable in that it includes chromatographic and spectroscopic data which can be used for the separation and identification of phytoalexins. In his comprehensive review of phytoalexin biosynthesis A. Stoessl separates the phytoalexins according to their biosynthetic origin from shikimic acid, acetate-polymalonate and mevalonate; he also emphasizes the value of stressed tissue for biosynthetic studies.

In the remaining contributions, comprising the second part of the book, the accent is on the possible roles of phytoalexins in plant diseases. The reactions involved in the metabolism of phytoalexins both by microorganisms and plants are described by H.D. VanEttten and colleagues, and D.A. Smith discusses the toxicity of phytoalexins and their metabolites. J.W. Mansfield then provides a detailed description of phytoalexin accumulation in the interaction of several leguminous and solanaceous plants with fungi, bacteria, viruses and nematodes. It is clear from these accounts that pathogenicity cannot necessarily be explained by the ability of the invading pathogen to tolerate or metabolize phytoalexins, although in many

cases of resistance there is sufficient accumulation of phytoalexins to account for this resistance; nevertheless other factors such as suberization and lignification must be taken into account.

In his review of the mechanisms of phytoalexin accumulation, J.A. Bailey indicates that the underlying mechanisms leading to the build-up of all classes of phytoalexin may be similar. He proposes that host cell death releases an endogenous elicitor which triggers the enzymes involved in phytoalexin biosynthesis, and points out that specificity would involve control of cell death. The final chapter, written by the two editors themselves, is an excellent summary of work on phytoalexin function to date and of possible ways in which it could proceed in the future.

The overall presentation, particularly of the formulae and diagrams, is good and there are very few errors. Moreover there is a comprehensive bibliography with each chapter, and a compound and an organism index at the end of the book (though, unfortunately, no author index). *Phytoalexins* will be the standard work on the subject for some considerable time; as such it is an essential reference volume for all workers in plant disease physiology and phytoalexin biosynthesis. □

John Friend is Professor and Head of the Department of Plant Biology at the University of Hull.

Flies in the lab

Michael Ashburner

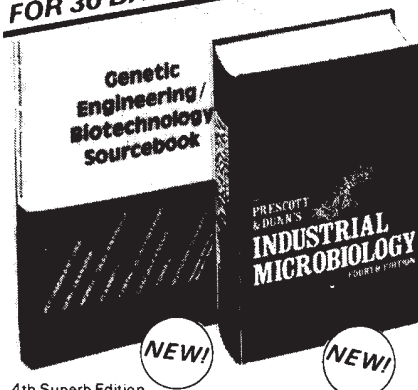
A Handbook of Drosophila Development. Edited by Robert Ransom. Pp.289. Hbk ISBN 0-444-80366-1; pbk ISBN 0-444-80418-8. (Elsevier Biomedical: 1982.) Hbk Dfl.250, \$116; pbk Dfl.100, \$46.

THE EDITOR of this collection of reviews on the development of *Drosophila* states his objective as being:

to provide a useful and practical research aid, which may be kept close at hand on the researcher or students [sic] own bookshelf.

This, however, is not a "handbook" in the German tradition; the fact that you can actually pick it up with one hand establishes that. Yet its chapters do provide useful, if not over-scholarly, reviews of the embryonic and imaginal development and structure of *Drosophila*. There is, of course, some overlap with Vol.2 of *The Genetics and Biology of Drosophila* (Academic, 1978) — a work in which I must declare some interest — but except for two

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chapters this is minimized by the differences in organization between the two works. I am disappointed, however, that this book has not been truer to its design, restricting itself to hard data and not degrading this information with speculative "noise". In each chapter the worst sections are those with some such heading as "Genetic studies on . . .". These contribute very little to what is already easily available and are, in general, too superficial to be of any interest or use.

An editor of a "handbook" has a duty to ensure that the factual information is correct. This ideal can never be wholly achieved, yet I cannot but warn the reader of this book that the editor's own chapter, on techniques (and to a lesser extent his contribution on internal organs), is seriously flawed.

Perhaps I should quote one or two errors of fact: "Each female only mates once". Quite untrue. "Sex is determined by X chromosome dosage". Again untrue, it is

determined (in *Drosophila*) by the X:autosome ratio. "This phenomenon [mitotic recombination] can be induced spontaneously". No comment.

Finally, I defy any reader to understand the mechanism of mitotic recombination from Fig. 6, which shows undivided centromeres as if this were the first division of meiosis; I also do not approve of the illustration of methods to screen for mutations by specialized, rather than more general techniques (i.e. Figs 11 and 12).

Happily, this standard is not maintained in the other chapters. There are one or two annoying features (for example Fig. 1 of Chapter 4 and Fig. 16 of Chapter 7 are identical), but in general the central eight chapters are solid and competent and make the book a useful addition to the *Drosophila* biologist's laboratory bookshelf. □

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Volume 2, *Physiology and Pharmacology*, covers, as it were, the applications of cyclic nucleotides in physiological processes. It gives an idea of their breadth of function, with topics ranging from slime moulds to the mammalian brain.

An interesting contrast emerges from these volumes between our knowledge of the role of cyclic AMP and that of cyclic GMP. Although cyclic GMP was discovered as long ago as 1963, its functions are still largely obscure. The Yin-Yang hypothesis, so delightfully named by Nelson Goldberg, in which cyclic GMP was seen as a messenger opposing the effects of cyclic AMP, now seems untenable. While Murad speculates here that cyclic GMP might be a general feedback regulator of redox state, it seems possible that despite its ubiquitous occurrence it in fact performs only specialized functions (for example in dark adaptation in the retina as discussed by Farber in Vol. II). This would be rather analogous to its parent nucleotide GTP which, in its guise as energy donor rather than nucleic acid precursor, has a small number of rather specialized roles one of which is the regulation of cyclic AMP formation by adenylate cyclase.

In his introduction to the two volumes, Rall states that the 1960s saw a great advance in our understanding of the mechanism of action of cyclic AMP in physiological systems, while in the 1970s the progress has been marginal. This is indeed the overall impression given by Vol. II, with a very few of the systems described being understood in molecular terms. However I believe this view to be misleading. It reflects, in part, an emphasis on systems with relevance to pharmacology where the basic processes themselves are not understood. It also ignores the fact that of the 20 or so physiological substrates for cyclic AMP-dependent protein kinase whose function is known, all but three have been identified since 1970. Given that these substrates are tabulated in the chapter on cyclic AMP-dependent protein kinases in Vol. I, some of the omissions in Vol. 2 are very surprising. For example, a chapter on "Regulation of Lipid Metabolism by Cyclic Nucleotides" makes no mention of any of the well-characterized phosphoproteins involved in lipid synthesis, such as acetyl-CoA carboxylase or hydroxymethylglutaryl reductase. And that entitled "The Role of Cyclic Nucleotides in the Vasculature" states that the causal link between cyclic AMP and the relaxation of vascular smooth muscle is unknown — the work of Adelstein and others on relaxation of smooth muscle via phosphorylation of myosin light-chain kinase is not mentioned. Readers who prefer their explanations in molecular terms, or at least in terms of defined proteins, will find Vol. II disappointing, and in my view this need not have been the case. □

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Twenty-five years of cyclic nucleotides

D.G. Hardie

Cyclic Nucleotides, Vols 1 and 2. Edited by J.W. Keibarian and J.A. Nathanson. Vol. 1 *Biochemistry*, pp. 557, ISBN 3-540-10786-X; Vol. 2 *Physiology and Pharmacology*, pp. 888, ISBN 3-540-11239-1. (Springer-Verlag: 1982.) Vol. 1 DM 390, \$173.20; Vol. 2 DM 560, \$248.70.

IT HAS been possible to show that the response of the homogenates to the hormones occurred in two stages. In the first stage, a particulate fraction of homogenates produced a heat-stable factor in the presence of the hormones; in the second stage, this factor stimulated the formation of liver phosphorylase in supernatant fractions of homogenates in which the hormones themselves were inactive [T.W. Rall, E.W. Sutherland and J. Berthet *J. Biol. Chem.* 224, 463–475, 1957].

This unpretentious announcement of the discovery of a heat-stable factor, later identified as cyclic AMP, quietly heralded the era of the cyclic nucleotides. While the authors no doubt realized that their observations represented a breakthrough, they can hardly have guessed at the impact they would ultimately have. Twenty-five years later it is clear that cyclic AMP has a

central role in cellular regulation, equivalent to that of its parent nucleotide ATP in metabolism.

These two volumes provide the latest review of the field and are, in the words of the editors, an attempt at a "comprehensive and up-to-date anthology" with contributions by 72 well-known cyclic nucleotide researchers. The books appear in the much-respected *Handbook of Experimental Pharmacology* series, to which all pharmacology departments no doubt subscribe. But do they deserve a wider audience?

The two volumes are to a certain extent independent of each other. Volume 1, *Biochemistry*, covers the enzymology of cyclic nucleotide metabolism and of the protein kinases which form the only known intracellular receptors for the cyclic nucleotides. It also provides ample discussion of the interactions between cyclic nucleotides and that other ubiquitous cellular regulator, the Ca^{2+} ion; calcium, at least in its free cytosolic version, seems now to have almost acquired the status of "honorary cyclic nucleotide". Volume 1 gives a well-balanced and reasonably up-to-date account of these aspects of the field. A notable omission, however, is any discussion of protein phosphatases, which do not even appear in the index. This is regrettable because it is now apparent that some actions of cyclic AMP are mediated through inhibition of protein phosphatase-1 by the heat-stable inhibitor protein which is phosphorylated by cyclic AMP-dependent protein kinase (a topic reviewed recently by Philip Cohen in *Nature* 296, 613–620; 1982).

● A second edition of *Germ Cells and Fertilization*, Book 1 in the series *Reproduction in Mammals*, has recently been published by Cambridge University Press. Prices are hbk £15, \$24.50, pbk £5.95, \$11.95.

Four further volumes in the second edition of the series (which is edited by C.R. Austin and R.V. Short) are scheduled to appear in due course.